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Droplet Electrospray Ionization Mass Spectrometry for High Throughput Screening for Enzyme Inhibitors

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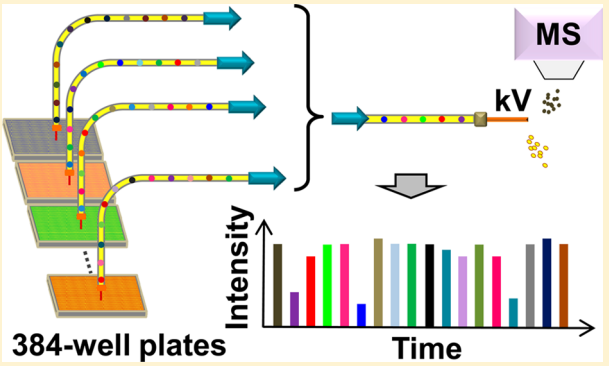
Supporting Information

ABSTRACT: High throughput screening (HTS) is important for identifying molecules with desired properties. Mass spectrometry (MS) is potentially powerful for label-free HTS due to its high sensitivity, speed, and resolution. Segmented flow, where samples are manipulated as droplets separated by an immiscible fluid, is an intriguing format for high throughput MS because it can be used to reliably and precisely manipulate nanoliter volumes and can be directly coupled to electrospray ionization (ESI) MS for rapid analysis. In this study, we describe a "MS Plate Reader" that couples standard multiwell plate HTS workflow to droplet ESI-MS. The MS plate reader can reformat 3072 samples from eight 384-well plates into nanoliter droplets segmented by an immiscible oil at 4.5 samples/s and sequentially analyze them by MS at 2 samples/s. Using the system, a label-free screen for cathepsin B modulators against 1280 chemicals was completed in 45 min with a high *Z*-factor (>0.72) and no false positives (24 of 24 hits confirmed). The assay revealed 11 structures not previously linked to cathepsin inhibition. For even larger scale screening, reformatting and analysis could be conducted simultaneously, which would enable more than 145 000 samples to be analyzed in 1 day.



High throughput screening (HTS) is important in drug discovery, chemical biology, and chemistry. Current technology relies mostly on performing assay reactions in multiwell plates (MWP) with robotic manipulation of fluids followed by interrogation using optical plate-readers.[1,2] Mass spectrometry (MS) is a potentially powerful technique for HTS because it is fast, has high resolution, and can detect chemicals without labels.[3,4] This latter advantage is important because it eliminates false signals based on a label or indicator reaction and avoids the time, expense, and expertise needed to modify target compounds for optical assay. Use of MS in screening[5−8] has often relied on the multiplexing capability to test mixtures of compounds; however, this use is limited because most chemical libraries are formatted as arrays of individual compounds and most screening is performed by testing one compound on one reaction at a time. MS is not commonly used for such screening because traditional sample introduction methods are too slow; although advances have been made with high flow rate separation and multiplexed autosamplers.[9,10] As we describe here, a way to overcome this limitation is use of segmented flow sample introduction to enable label-free HTS by electrospray ionization (ESI)-MS.

Segmented flow and other formats wherein aqueous sample droplets are compartmentalized within an oil carrier fluid have seen a resurgence of interest due to advances in microfluidic manipulation tools.[11−16] Although most droplet experiments rely on optical detection, ESI-MS can also be used to analyze droplets.[17−23] Droplet sample introduction for MS allows fast analysis and greatly reduced sample consumption, suggesting potential for HTS. Despite this potential, no reports have demonstrated the robustness needed for screening many compounds. Instead, experiments have focused on methods of interfacing droplets to MS or small scale screens of a few compounds under conditions not compatible with HTS. A potential approach to HTS by droplet MS is to complete entire screening reactions at the droplet level yielding both label-free detection and miniaturization.[18] However, a substantial infrastructure investment in MWP-based technology suggests that it is also of interest to combine such tools with MS, i.e., to develop a "MS plate reader". Herein we describe coupling MWP-based fluid manipulation with segmented flow ESI-MS to rapidly screen a compound library. The approach utilizes a two-step process of reformatting MWP samples into droplets and then infusion into ESI-MS.

The system is applied to cathepsin B,[24] a cysteine protease implicated in tumorigenesis, arthritis, and parasite infection.[25−28] Both in vivo and in vitro studies have demonstrated that certain cathepsin B inhibitors reduce tumor cell motility and invasiveness.[29,30] Because of these links, considerable effort has been made to identify cathepsin B inhibitors. Successful

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inhibitors include epoxysuccinyl, aziridinyl, biguanide, and β-lactam derivatives.[31,32] The MS screen used here reveals several potential new inhibitors.

EXPERIMENTAL SECTION

Chemicals and Materials. Unless otherwise specified, all solvents were purchased from Honeywell Burdick & Jackson (Muskegon, MI) and were certified ACS grade or better. Reagents were purchased from Sigma-Aldrich (St. Louis, MO). Peptides used for cathepsin B assay were synthesized by Pierce Biotechnology, Inc. (Rockford, IL). Compounds for cathepsin B screening were from Prestwick Chemical Library (Prestwick Chemical, Washington, DC), provided by the Center of Chemical Genomics of University of Michigan.

Parallel Droplet Generation. Oil-segmented droplets of 50 nL each were created in parallel from eight 384-well plates into eight 0.01 in. i.d. × 1/16 in. o.d. fluorinated ethylene propylene (FEP) tubes (IDEX Health and Science, Oak, Harbor, WA). A 2 cm 100 μm i.d. × 238 μm o.d. fused-silica capillary, sealed with Sticky Wax (KerrLab, Orange, CA), was inserted into the inlet of each FEP tube to act as a "sipper". Capillaries were fluorinated by pumping 1:100 (v/v) trichloro(1H,1*H*,2H,2*H*-perfluorooctyl)-silane in anhydrous hexadecane through them. Samples in 384-well plates (Nunc 384-Well ShallowWell plates, Thermo Scientific) were covered with perfluorodecalin (PFD, 95% purity, Acros Organics, NJ). The edges of plates were built-up to 5 mm height with epoxy to hold PFD over the wells.

For droplet formation, the 384-well plates and the inlets of the FEP tubes were mounted onto a computer numerical control (CNC) machine (Cameron Micro Drill Press, Sonora, CA) so that a sipper was above the first well on each plate. The other end of FEP tubes were connected to 500 μL Hamilton syringes (Fisher Scientific, Pittsburgh, PA), which were mounted on a multichannel syringe pump (Fusion 400, Chemyx Inc. Stafford, TX). The syringes and FEP tubes were prefilled with PFD. As the syringes were aspirating at 4 μL/min, the G-code programmed CNC machine controlled the movement of tubes and plates so that the sipper could alternatively dwell in sample for 1 s and in oil for 0.25 s, as well as move from sample to sample for 0.5 s. Droplets with 50 nL volume separated by equal size of oil were produced with these parameters.

Mass Spectrometry Analysis. A Micromass Quattro Ultima triple quadrupole MS (Waters Corporation, Milford, MA) was used for analysis. The original stainless steel ESI needle was replaced by a piece of fused silica capillary (18 cm in length, 100 μm i.d. × 238 μm o.d.) coated on the outside with gold and with the inner surface fluorinated as described above for sipper capillaries. The FEP tube containing sample droplets was connected to the treated ESI needle with a 1/16 in. bore VICI Cheminert union (Valco Instruments Co. Inc. Houston, TX).

For analysis, a syringe pump drove sample droplets through the needle into the source. ESI voltage was +2.5 to 3.0 kV, the source was heated to 100 °C, the cone gas was set at 50 L/h, the desolvation gas was 200 L/h, and the nebulizing gas was adjusted to the best flow based on the infusing rate of droplets. The ESI mode and the MS method were dependent upon molecules to be analyzed. Extracted ion currents (XICs) of target molecules were obtained by MassLynx (Version 4.1, Waters Inc.). Sample droplets are detected as bursts of current in the XICs because the oil, being not conductive or charged, does not generate an ESI-MS signal.

Cathepsin B Assay. The assay was developed starting from a previous report.[18] The nonfluorogenic peptide GFGFVGG was used as the substrate for cathepsin, which yields FVGG as a product (Figure S3a, Supporting Information). Reactions were performed in 20 mM ammonium formate and 200 μM 1,3-dithioerythritol (DTE) buffer. Reactions were stopped with equal volume of quenchant consisting of ice-cold 50% methanol, 50% water, and 0.3% formic acid (v/v). The quenchant also contained a 20 μM stable isotope labeled product (FVG*G, +3 Da) as the internal standard. The buffer is MS compatible, which allows direct infusion analysis. To determine the linearity of the reaction rate, assays containing final concentrations of 50 nM cathepsin B and 100 μM substrate were incubated for 0, 15, 25, 35, 45, 65, and 90 min. To determine Michaelis−Menten kinetics, the substrate concentration was varied from 0 to 400 μM while quenching the reaction at different time points from 0 to 90 min. The K_m value was fit by Michaelis−Menten model using GraphPad Prism 6.01.

High Throughput Cathepsin B Inhibitor Screening. To screen the Prestwick Library, 8 μL of 100 μM GFGFVGG was deposited into each well of four 384-well standard assay plates (Greiner Bio-One, Monroe, NC) by Multidrop Combi (Thermo Scientific, Waltham, MA). 50 nL of 5 mM test compounds from the Prestwick Library (1280 chemicals) was then added with a Caliper Life Sciences Sciclone ALH 3000 Workstation (PerkinElmer, Waltham, MA). Two microliters of 0.25 μM cathepsin B was deposited into the mixture afterward. The final concentrations were 80 μM GFGFVGG, 50 nM cathepsin B, and 25 μM test compound. 0.5% Dimethyl sulfoxide (DMSO) was present in each reaction. After incubation at 37 °C for 25 min, reactions were quenched with 10 μL of ice-cold quenchant. In total, 1408 reactions, including 64 negative controls (DMSO) and 64 positive controls (25 μM E-64), were performed. Assay plates were then spun to remove air bubbles. All mixtures were finally transferred into 384-well readout plates with elevated edges by a Biomek FX^P Laboratry Automation Workstation (Beckman Coulter, Brea, CA). Samples were reformatted to droplets as described above. Each sample was collected as 3 droplets in sequence. Four FEP tubes of 1 m length were used for each plate. Droplet and oil gap had equal volume of 75 nL. The analysis was performed using ESI-MS in multiple reaction monitoring with m/z transition 379.5→247.1 for FVGG and 382.5→247.1 for FVG*G. The collision energy was set as 18 eV, dwell time was 0.01 s, and interchannel delay was 0.01 s for both transitions. The ESI voltage was +2.7 kV. Droplets in FEP tubes were pumped into the sample cone at 15 μL/min. Droplet traces were acquired by MassLynx and processed with Origin 8.5.

Hits Validation. Inhibitor hits were tested by dose dependent experiments, which were performed under the same condition as the screening. Each reaction contained a test compound from 0.1 μM to 100 μM (unless shown otherwise on the data plots). Peak height ratio of the product FVGG and the isotopic standard FVG*G were used for analysis. The data were normalized to a control reaction that contained no inhibitor. Such normalization accounted for variation in reaction yield seen from day to day, possibly due to enzyme variation during storage. Fluorescent assays were also performed (see details in the Supporting Information) to corroborate our finding. IC_{50} values were obtained from dose response curves, which were fitted by GraphPad Prism 6.01.

RESULTS AND DISCUSSION

Figure 1 illustrates the two-step process for plate reading by segmented flow ESI-MS. Samples are first reformatted from a MWP to droplets arranged sequentially in a fluorinated ethylene

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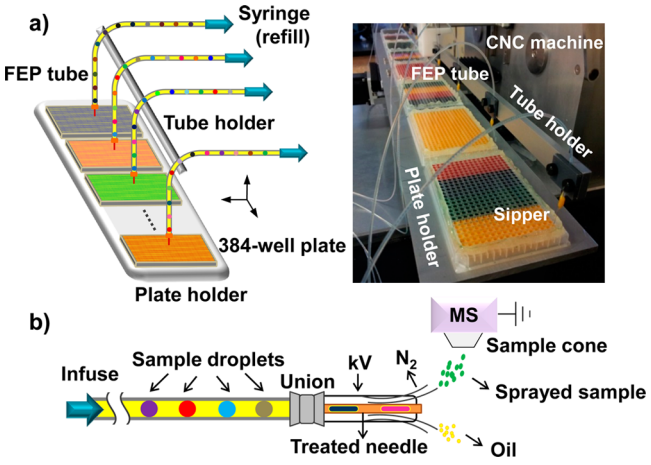

Figure 1. (a) Left: scheme of parallel oil-segmented droplet generation from MWPs. FEP tubes are programmed to dwell in sample or oil for predetermined time, and then move to another well. The syringe pump operates in refill mode at a constant flow rate. Right: picture of parallel droplets generation with different color food dye as samples. (b) Diagram of ESI-MS analysis by direct infusion of segmented flow. Droplets are pumped into the ESI source through a treated ESI needle. ESI voltage is applied on the needle. In the gas phase, charged sample droplets (green) enter the MS and nebulized oil (yellow) does not.

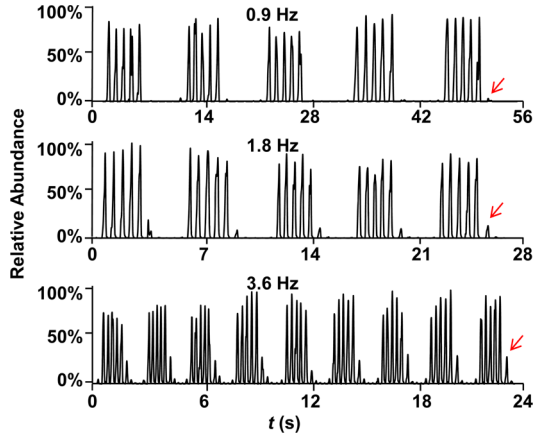

Figure 2. Carry-over evaluation at different analysis rate. Five micromolar adenosine and solvent (20% methanol, 0.1% formic acid) alternating droplets (5 each) were infused into the ESI source for analysis. At 0.9 Hz, the carry-over is almost zero; at 1.8 Hz, it increases to 10%; at 3.6 Hz, it becomes as high as 20−30%. Red arrows indicate solvent droplet that contains signal due to carry-over.

propylene (FEP) tube and segmented by perfluorodecalin (PFD) carrier fluid. The segmented array of samples are then pumped directly into a metal-coated fused silica capillary that acts as the ESI needle (Figure 1b).[19] The inner surface of the capillary is fluorinated so that it is wetted by the PFD. ESI voltage is applied continuously but electrospray stops and starts with each aqueous plug that exits the channel. The off-axis MS inlet prevents PFD, which is nebulized but does not form charged droplets, from entering the MS inlet (Figure 1b); thus a separation of oil and sample occurs in the gas phase. Mass spectra obtained for segmented samples are comparable to direct infusion and we do not detect signals that can be attributed to PFD at any time during infusion of samples (Figure S1, Supporting Information), suggesting effective separation and no detrimental effects of oil on the MS performance.

Rate of analysis by ESI-MS. We first examined the rate of ESI-MS possible. The rate of mass spectra acquisition sets the ultimate limit for analysis rate. The highest scan rate of the MS used for this work is 62 scans/s for single reaction monitoring and 27 scans/s for monitoring two reactions. For quantitative analysis, it was desirable to obtain 6−8 scans per droplet, which means that the analysis rate can be up to 5 Hz (i.e., 5 samples/s) for measuring a single ion and 2 Hz for two ions (assuming a 1:1 ratio of sample:oil). For 50 nL droplets, the MS-limited rate of 5 Hz could be achieved with an infusion flow rate of 30 μL/min. This rate produced stable traces and reproducible detection of a select ion showing that the ESI can stop and start at such rates. We could reliably infuse up to 500 droplets at a time, the largest read length tested.

Analysis rate is also affected by the amount of carry-over that can be tolerated because we found a link between carry-over in the MS signals and analysis rate. For example, for 5 μM adenosine followed by a blank droplet we detected no carry-over at 1 sample/s; but 10% at 2 samples/s and 20−30% at 4 samples/s (Figure 2). We observed no cross-contamination between droplets within the storage tubes as visualized by dyes, presumably due to low partition coefficients into the carrier fluid and wetting of the FEP surface by PFD. Therefore, the carry-over may be due to cross-droplet contamination occurring during transfer from FEP to ESI needle, within the needle, or in the gas phase. With the present system, carry-over can be reduced by decreasing the analysis rate or by introducing replicate samples. In the latter case, contamination becomes negligible after the first droplet so that averaging the signal from 3 droplets gives good quantification. Use of triplicate samples also provides redundancy for cases where a noise spike affects a measurement.

Rate of Droplet Formation. It is necessary to have rapid reformatting from MWP to droplets for overall high throughput. Previous reports demonstrated up to 0.15 droplets/s for forming segmented arrays from MWP, which would be rate limiting in this case;[33] therefore, we explored increasing the rate of this step. Reformatting followed the general procedure previously described for PCR in droplets.[34] In this method, samples in a MWP are covered with a continuous layer of oil. The tip of a FEP tube, connected at the opposite end to a syringe operated in withdraw mode, is moved from well-to-well to generate samples separated by oil segments. Fast movement and high aspiration rate contribute to high droplet generation rate. Using an aspiration rate of 4 μL/min allowed at least 400 droplets of 50 nL to be formed inside a 90 cm long tube at a rate of 0.58 Hz and a droplet size relative standard deviation (RSD) <5% (Figure S2, Supporting Information). A higher aspiration rate tended to produce progressively smaller droplets as the tube was filled, likely due to increased leaking as the flow resistance increased with more droplets. To prevent droplet generation from being rate limiting, tubes can be operated in parallel. For example, we operated 8 tubes simultaneously to give an overall sample generation rate of 4.5 Hz with an RSD of droplet size across tubes <10% (Figure S2, Supporting Information).

Screening. Although we have previously demonstrated an MS assay for cathepsin B,[18] it was necessary to identify appropriate conditions and validate the assay for HTS. The assay used the heptapeptide GFGFVGG (Figure S3, Supporting Information), a sequence representing the proteolytic preference of cathepsin B, as the substrate.[35] We elected to monitor the product peptide (FVGG) as increases on its low background were easier to measure than decreases in the substrate peptide. A stable-isotope labeled product peptide was added to the assay

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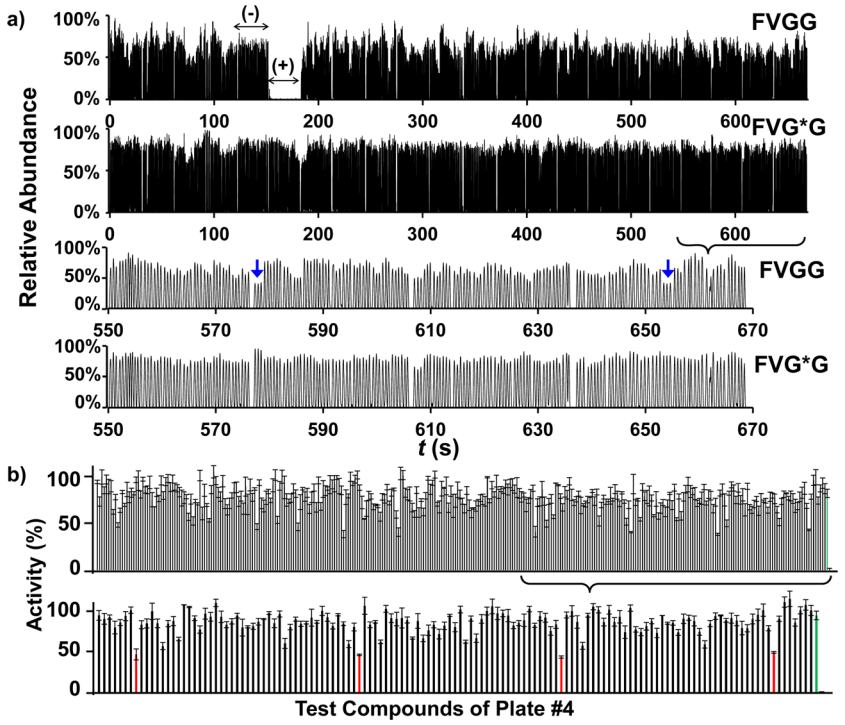

Figure 3. (a) Top: droplet traces of partial cathepsin B inhibitor screening (Plate #4, 320 test compounds, 16 negative controls (−), and 16 positive controls (+)). Each reaction is analyzed in triplicate. The analysis rate is 1.6 Hz (1056 droplets detected in 670 s). Bottom: enlarged view of 550−670 s. Inhibitors (blue arrows) are identified by the low intensity ratio of FVGG/FVG*G. (b) Top: the analysis result of Plate #4. Each bar is the averaged FVGG/FVG*G of an assay. The negative control is normalized to 100% activity. Bottom: last 135 reactions and controls (green). Inhibitors (red) are identified by the low % of activity (n = 3).

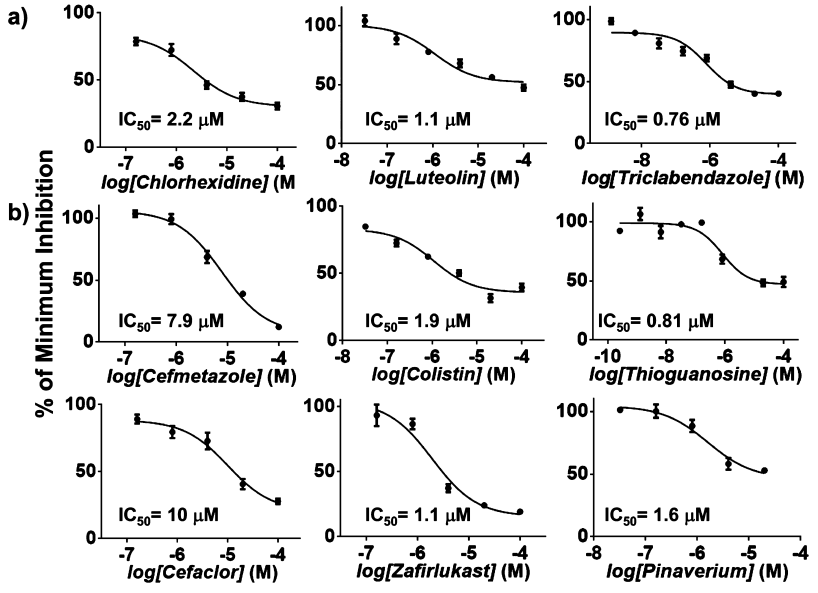

Figure 4. Dose response curves of some of the identified inhibitors. (a) Drugs proved to inhibit cathepsins or treat cathepsin-related diseases.[42] The reported IC_{50} of chlorhexidine is 40 μM.[37] The reported inhibition efficiency of luteolin is reducing the activity of cathepsin B by 60% at 40 μM.[38] (b) Drugs that reduced the yield >40% in the primary screen but had no previous link to cathepsins (n = 3).

mixture as an internal standard, so that any changes in ionization efficiency caused by test compounds or drift in the MS signal would not affect quantification of the reaction. Calibration curves for detection of product peptide, measured as a ratio to the internal standard, were linear from 0 to 500 μM in the quenched assay solvent (Figure S3c, Supporting Information). The rate of product formation was linear for 60 min when using 50 nM cathepsin B and 100 μM substrate (Figure S3d, Supporting Information). Michaelis−Menten analysis yielded a K_m of 270 ± 34 μM (Figure S3e, Supporting Information). Based on the kinetic studies, each screening reaction contained a final concentration of 50 nM cathepsin B and 80 μM substrate, below the K_m, and was incubated for 25 min to ensure linear reaction velocity. These conditions satisfy the requirements for HTS.[1,36]

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Assay conditions were validated with a 24-compound pilot screen including 4 known inhibitors: E-64 (L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine), leupeptin (acetyl-Leu-Leu-Arg-al, N-acetyl-L-leucyl-L-leucyl-L-argininal), antipain [(*S*)-1-carboxy-2-phenylethyl]carbamoyl-L-arginyl-L-valyl-argininal) and chymostatin (N-(Nα-carbonyl-[*S*,*S*]-α-(2-iminohexahydro-4-pyrimidyl)glycine-X-Phe-al)-Phe, X = Leu, Val, or Ile) at 25 μM each. All of these compounds were detected as inhibitors in the pilot screen and dose response curves of those 4 inhibitors yielded expected IC_{50} values (Figure S4, Supporting Information). These results suggest that the assay conditions do not adversely affect kinetics of the reaction or performance of known inhibitors.

We used this system to screen the Prestwick Chemical Library, which consists of 1280 FDA-approved drugs, against cathepsin B. The screen used standard MWP fluid manipulation for creating the reaction mixtures in 384-well plates and therefore can be incorporated into existing MWP screening systems. Performing the assay reaction, including reagent dispensing, incubation, and transferring to detection plates, took 1 h. The 1408 reaction mixtures (1280 test compounds and 128 controls) were reformatted into 4224 droplets of 70 ± 7 nL. Droplets were generated at 0.5 Hz/tube and MS analysis was conducted continuously at 1.6 Hz yielding stable signals (Figures 3 and S5, Supporting Information). The analysis of all sample droplets took ∼45 min. Carry-over was lower than 15% from droplet to droplet during the screen, which did not affect the analysis of triplicate samples (Figure S5, Supporting Information). The assay was robust, as the *Z*-factors of all plates were above 0.72. Although adding detection of isotope-labeled internal standard required a slower MS scan rate, it was necessary to correct ion suppression and signal drift. For example, reanalysis of the data revealed that if internal standard had not been used, 3 hits would be missed and 12 noninhibitors would be assigned as hits because of such signal variations.

Among 1280 test compounds, 9 reduced the reaction yield more than 50% and 15 inhibited the reaction ∼40%. Interestingly, all hits were confirmed by dose response assays (Figures 4 and S6, Supporting Information) showing a low false positive rate. The dose response curves all had R^2 values of 0.92 or better, indicating reasonable fits to the expected sigmoid. To further validate the results, we performed fluorescent assays on several of these hits to test results in an orthogonal assay (Figure S7, Supporting Information). The tested compounds all yielded dose response curves in both assays with similar IC_{50} values. Variation may be attributed at least in part to use of different substrates for the assays.

Of the 24 chemicals that reduced the reaction yield 40% or more, 4 were known cathepsin or cysteine proteases inhibitors (chlorhexidine, luteolin, ethacrynic acid, and disulfiram). Their inhibition efficiency is comparable to the published values.[37−39] Another 6 hits (triclabendazole, hexachlorophene, anthralin, raloxifene, triclosan, and diacerein) have not been reported as cathepsin inhibitors, but are used to treat diseases in which cathepsins play a role. For example, triclabendazole is used to treat liver flukes, which secrete cathepsins.[40,41]

The cathepsin inhibitory effect of the other 14 hits has yet to be studied, which suggests potential new therapeutic use for these compounds. Among them, 3 contain known "warheads" of small molecule cathepsin B inhibitors (cefmetazole and cefaclor are β-lactams and alexidine contains a biguanide[32]). The remaining 11 hits do not contain structural motifs established as cathepsin B inhibitors, suggesting an opportunity to develop modulators based on novel moieties.

Higher Throughput. In principle, this system can sustain high throughput for a large number of samples. For example, 3072 droplets can be created from eight 384-well plates within 12 min and analyzed in 26 min. Switching tubes from aspiration to MS manually required 4 min for 8 tubes. By overlapping droplet generation of the new batch with the analysis of the previous one, 147 456 droplets could be analyzed in 24 h, suggesting the potential for ultrahigh throughput MS analysis. The switching of tubes could be automated for faster analysis. Improving the read length could also reduce the number of times that this step is required further improving throughput.

Comparison to Other MS Screens. MS has emerged as a useful tool for HTS in a variety of ways. Some approaches take advantage of multiplexing to screen many compounds in one reaction and use MS to help sort the results.[5−8] Although multiplexing was not demonstrated here, the rapid readout of the droplet-based approach could be used to help further speed up multiplexed assays. Most ESI-MS-based screening systems incorporate liquid chromatography or solid-phase extraction.[9,43,47] These systems have reported assay rates up to 1 sample/8 s, thus, the droplet ESI-MS method here can operate at over 10 times higher throughput, but is restricted to assays that can be run in solvents that are compatible with ESI-MS. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has also been used for screening. The sample preparation is relatively easier and a higher level salt can be tolerated. The throughput of MALDI-based assay is typically around 1−2 min.[44−46]

CONCLUSION

These results demonstrate that droplet ESI-MS has the robustness and throughput to be used for HTS applications. The system provided reliable results for over 4000 samples in a HTS workflow. The low false positives and identification of novel compounds support the idea that high-throughput, droplet-based ESI-MS can be a powerful tool for label-free screening. Although the current droplet system has higher throughput than systems that use solid-phase extraction or LC,[43,47] it is restricted to assays that can be performed in ESI-MS compatible buffers. Although many targets will likely be compatible with such buffers, this limitation does suggest that future work should be directed toward methods of rapid sample cleanup, e.g., parallel solid-phase microextraction[48] or in-plate solid-phase extraction[49,50] so that the high throughput capability could be used with a wider range of assays. Another important advantage relative to current MS-based methods is that this approach uses miniscule fractions of a sample and therefore could be compatible with lower volume, higher density well plates for reduction of reagent consumption. Overall, these results suggest that the droplet-based method adds to or complements existing MS screening systems.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest: R.T.K. has partial ownership of a company that uses droplet ESI-MS technology.

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