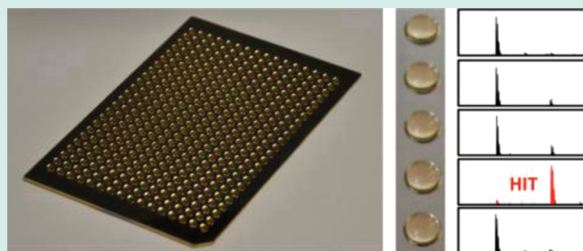# High-Throughput Screening of Small Molecule Libraries using SAMDI Mass Spectrometry

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**S** *Supporting Information*

**ABSTRACT:** High-throughput screening is a common strategy used to identify compounds that modulate biochemical activities, but many approaches depend on cumbersome fluorescent reporters or antibodies and often produce false-positive hits. The development of "label-free" assays addresses many of these limitations, but current approaches still lack the throughput needed for applications in drug discovery. This paper describes a high-throughput, label-free assay that combines self-assembled monolayers with mass spectrometry, in a technique called SAMDI, as a tool for screening libraries of 100 000 compounds in one day. This method is fast, has high discrimination, and is amenable to a broad range of chemical and biological applications.

**KEYWORDS:** histone deacetylase, high-throughput screening, SAMDI, mass spectrometry, self-assembled monolayers

High-throughput screening (HTS) is an important tool for identifying molecules that modulate enzymatic activities and is particularly important in the development of reagents used in biological research and the generation of hit compounds that initiate drug development programs.[1] Most HTS strategies rely on fluorescent reporter assays because these labels can be detected rapidly and with great sensitivity, and a variety of reagents are available that allow the fluorescent properties of the assay to be optimized.[2] Yet, it is well recognized that the labels can perturb enzyme activities, leading to a greater number of false positive signals,[3] and the development of a robust assay can require months of effort. Label-free strategies, including those based on refractive index properties[4] and mass spectrometry (MS),[5] have attracted wide interest since they can be applied to a broader range of assays using a common format, and they can be more rapidly developed and more robust since they eliminate the steps that are associated with installing and observing the label.

MS methods are particularly attractive because they report the mass of the molecule and therefore provide specific information on the analyte. Unlike many label-dependent formats, MS methods can observe each of the species present in a reaction, including the substrate for an enzyme, the intended products and unintended components, and therefore provide a more complete assessment of activity. Yet, MS methods have the drawback that they often require tedious protocols for sample preparation that decrease the throughput of the assay and significantly increase the cost. Methods that simplify sample preparation while maintaining a high-throughput capacity would address this need. We previously reported a MS technique that combines self-assembled monolayers (SAMs) of alkanethiolates on gold with matrix assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS, a technique termed SAMDI, for assays of enzyme activities.[6]

The substrates are immobilized onto SAMs in an array format either before or after treatment with an enzyme, and the surfaces are simply rinsed to remove salts and other components in the reactions, treated with matrix, and analyzed with a commercial MALDI-TOF instrument. Upon irradiation with the laser, the monolayers are efficiently desorbed from the surface through cleavage of the thiolate-gold bond and ionized, but undergo little fragmentation, to give relatively simple spectra that reflect the masses of the alkanethiolates and corresponding disulfides (Figure 1).[6] The SAMDI method has been applied to assays of kinases, proteases, glycosyltransferases, methyltransferases,[7] lysine deacetylases,[8] and nucleic acid ligases.[9] An early report also described the use of SAMDI to screen 10 000 small molecules to identify inhibitors of the anthrax lethal factor.[10] In that report, the screen used glass slides having an array of 10 by 10 circular grooves, each 2 mm in diameter. Each step in the screen was performed manually and several weeks were required to perform and analyze all the reactions.

In this paper, we describe the translation of SAMDI to a high-throughput format that can screen a 100 000 compound library in 24 h. This enormous increase in throughput follows from the fabrication and use of high-density SAMDI arrays and laboratory automation equipment to perform and analyze the reactions. The mass spectrometry is performed on stainless steel 'array plates' that are modified with gold circles that are each 2.8 mm in diameter and are arranged in the standard geometry of a 384-well microtiter plate. We prepare the array plates by sequentially washing the stainless steel substrates in individual baths of
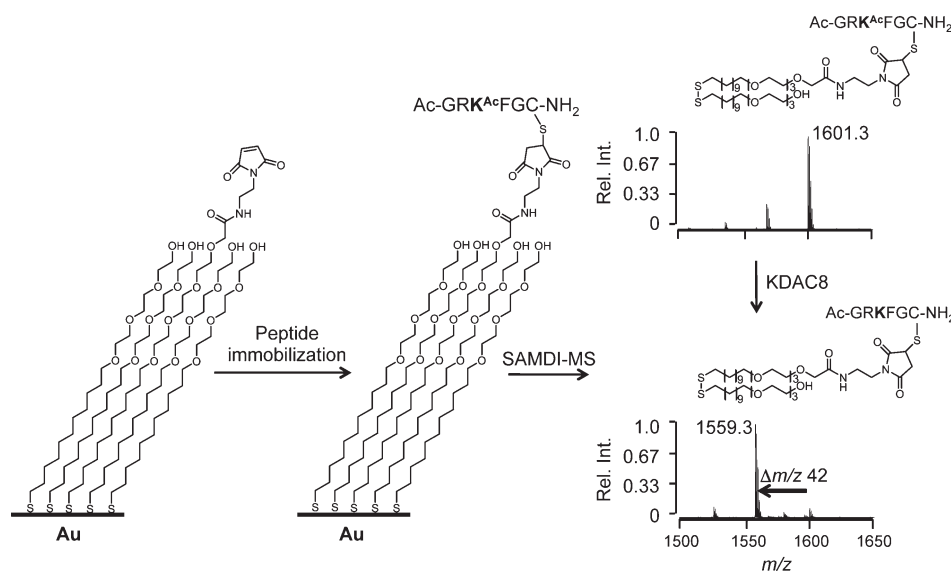
**Figure 1.** Schematic illustration of the SAMDI assay. Maleimide-terminated self-assembled monolayers are used to covalently capture the peptide substrates and products through reaction with the cysteine thiol group. Laser irradiation of the monolayer results in cleavage of the thiolate−gold bond and efficient desorption and ionization of the chains, either as disulfides or alkanethiolates. The starting peptide substrate used in the screen gives rise to the peak at a mass to charge ratio ($m/z$) of 1601.3, and the deacetylated products are shifted by $m/z$ 42 to a peak at $m/z$ 1559.3.

hexanes, ethanol, water, ethanol again, and then drying with nitrogen gas. An electron beam evaporator is then used to deposit a 10 nm layer of titanium on the entire plate, which will form a thin oxide layer as it is exposed to air during the 20 min that the evaporator is vented to ambient pressure. The plate is then covered with a mask and again placed in the evaporator to deposit titanium (4 nm) and then gold (22 nm) to create the array of gold features. The plates are then immersed in an ethanolic solution of terminally substituted disulfide reagents to assemble maleimide-terminated monolayers on each of the gold circles, as previously reported.[11] Finally, the plates are treated with hexadecyl phosphonic acid which reacts specifically with the titanium oxide surface surrounding the gold circles.[12,13] This treatment provides a hydrophobic surface that prevents the spreading of aqueous solutions from the monolayers, thereby reducing the potential for cross-contamination of the reactions.

We illustrate this approach with a screen of 100 000 compounds to identify inhibitors of lysine deacetylase 8 (KDAC8). KDACs (formerly histone deacetylases or HDACs) remove acetyl moieties from the $N^\varepsilon$-amino group of lysine residues and play a role in regulating gene expression, diabetes, and cancer and therefore have become popular therapeutic targets.[14] We also compare the SAMDI method to a common fluorescent format that uses the commercially available Fluor de Lys (FdL) KDAC assay.[15]

In preparation for screening, we organized a compound library (Chembridge Diverset) into pools of eight molecules (in DMSO) in 384-well plates. For all liquid transfers, we used a Tecan EVO liquid handler with a 96-tip head. A total of 320 wells in each plate had compound pools while the remaining wells (the first two and last two columns) were used for controls. Forty plates, each comprising 2560 compounds, were used to organize a total of 102 400 compounds. Reactions were performed in 384-well plates by adding 16 $\mu$L of buffer (25 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl$_2$) containing enzyme (1 $\mu$M KDAC8 final concentration) to each well. Next, the pooled compounds were transferred to the reaction plates and mixed

(2 $\mu$L, to give a final concentration of 1.25 $\mu$M for each molecule). The reactions were initiated by adding the KDAC8 peptide substrate Ac-GRK$^{Ac}$FGC-NH$_2$[8] (2 $\mu$L to give a final concentration of 10 $\mu$M), and the plate was incubated at 37 °C for 60 min.

The reaction mixtures (4.5 $\mu$L each) were then transferred onto the array plates (Figure 2a) and kept at room temperature in a humidified chamber for 1 h to allow the substrate/product peptides to immobilize to the maleimide-terminated monolayers. We verified that both the acetylated and deacetylated forms of the peptide underwent immobilization at a comparable rate (data not shown). The array plates were then rinsed with deionized ultrafiltered water, ethanol, dried with nitrogen, and treated with matrix (trihydroxyacetophenone, 50 mg mL$^{-1}$ in acetone). Each plate was analyzed with an Applied Biosystems 4800 MALDI TOF/TOF spectrometer using an automated protocol (20 kV accelerating voltage, positive reflector mode, 200 laser shots per spot), a process that requires approximately 4 s per spot. As shown in Figure 1, representative spectra from before and after enzyme treatment reveal two distinct peaks. The observed peak at $m/z$ 1601.3 corresponds to an alkyldisulfide of the acetylated peptide conjugated to the maleimide-terminated monolayer, and the peak at $m/z$ 1559.3 corresponds to the deacetylated form of this peptide-conjugated disulfide. The data (representative spectra shown in Figure 2b) were analyzed in an automated fashion using the Applied Biosystems Data Explorer Software to retrieve the area under curves (AUCs) that correspond to the deacetylated peptide and starting substrate. A parameter representing the extent of reaction was calculated using the relation $AUC_{Product}/[AUC_{Substrate} + AUC_{Product}]$ for each of the spectra. We found that 41 of the pools displayed a greater than 30% level of inhibition relative to control reactions that had no inhibitor present (Supporting Information Figure 1a). We then evaluated each of the 328 individual compounds and found that 48 of them showed greater than 50% inhibition (Supporting Information Figure 1b), and we identified one compound which gave nearly complete inhibition and had an IC$_{50}$ value of 200 nM (Figure 2c). We are currently working to further characterize the activity and

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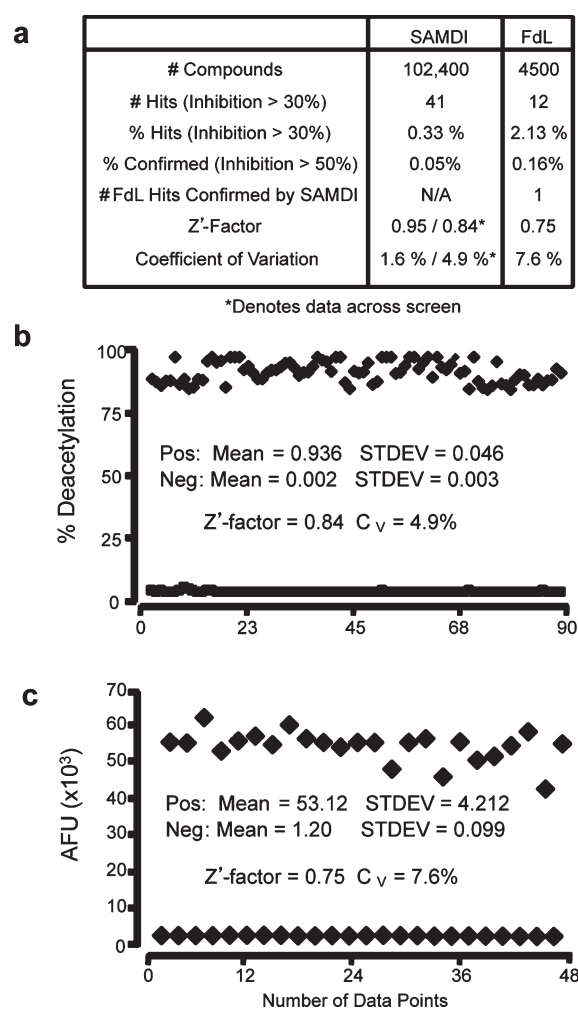
**a**



**b**



**c**
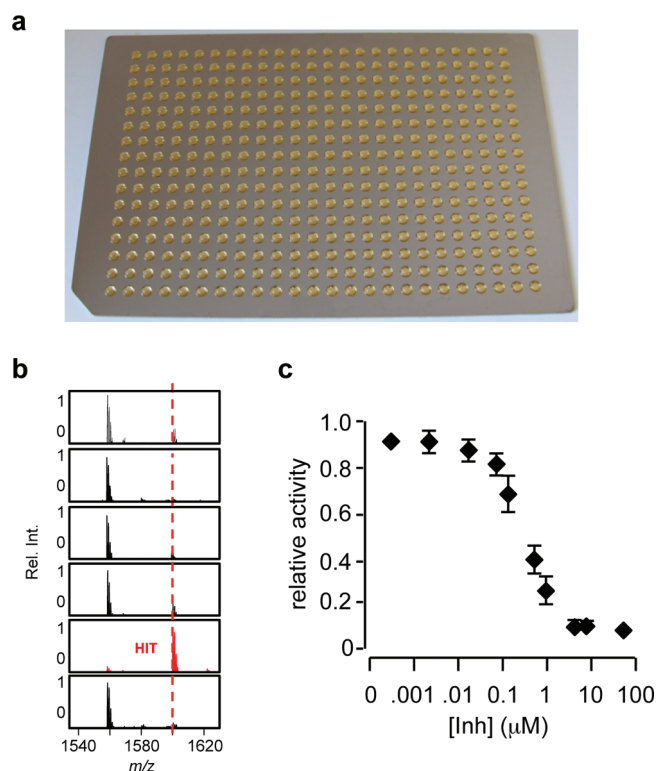


**Figure 2.** Combination of SAMDI-MS and peptide arrays gives a high-throughput screening method. (a) Image of a stainless steel array plate having an array of gold features in the geometry of a 384-microtiter plate. (b) Representative series of SAMDI spectra from the screen where the black spectra indicate deacetylated substrates and the red spectrum indicates the parent peptide, and therefore an initial hit. (c) Inhibition curve for our lead compound having an $IC_{50}$ value of 200 nM.

**a**

|  | SAMDI | FdL |
|---|---|---|
| # Compounds | 102,400 | 4500 |
| # Hits (Inhibition > 30%) | 41 | 12 |
| % Hits (Inhibition > 30%) | 0.33 % | 2.13 % |
| % Confirmed (Inhibition > 50%) | 0.05% | 0.16% |
| # FdL Hits Confirmed by SAMDI | N/A | 1 |
| Z'-Factor | 0.95 / 0.84* | 0.75 |
| Coefficient of Variation | 1.6 % / 4.9 %* | 7.6 % |

*Denotes data across screen

**b**



**c**



**Figure 3.** Comparison of SAMDI and Fluor de Lys assays as high-throughput screening tools. (a) Table of the details of each screen. (b) $Z'$-factor calculation of the SAMDI assay across the screen. ($C_V$ = coefficient of variation for the positive control, where $C_V$ = standard deviation/mean). (c) $Z'$-factor calculation for Fluor de Lys assay across one 96-well plate.

selectivity of this compound and corresponding derivatives with other KDAC isoforms and in cell culture.

In total, the screen was completed in 24 h. Individually, it took 6 h to prepare and immobilize the reactions to the array plates, 18 h to acquire 15 360 individual SAMDI spectra (~4 s/spot), and 30 min to process and evaluate the data, although many steps were performed concurrently. The secondary screen to deconvolute the mixtures was then completed in 3.5 h.

We also used the Fluor de Lys (FdL) assay kit (Enzo Life Sci. Inc.) to analyze 4500 randomly selected compounds from the same library, which is a sufficient number of compounds to allow a comparison of the two screening assays (Figure 3a). The assays were performed according to the manufacturer's protocol in a 96-well assay plate using KDAC8 at a concentration of 1 $\mu$M. The pooled compounds, at a final concentration of 1.25 $\mu$M, were added to each well, and control wells included the known pan-KDAC inhibitor Trichostatin A (TSA). We identified twelve pools (96 compounds, ~2% of the total) that showed more than 30% inhibition of KDAC8 activity (Supporting Information Figure 2a). We then used the FdL assay to evaluate the 96 individual compounds from these pools and identified seven compounds that inhibited KDAC8 activity by greater than 50% (Supporting Information Figure 2b). The final hit rate (defined as the percentage of compounds showing inhibition greater than 50% relative to the total number of compounds) was 0.16% for the FdL assay, whereas with SAMDI, we obtained a hit rate of 0.05%. Interestingly, when we evaluated the seven potent hit

compounds identified in the FdL assay with the SAMDI assay, we found that only one showed inhibition, suggesting that the remaining six compounds are false-positive hits. This result is consistent with a recent observation that fluorescently labeled substrates can lead to false-positive hits in screens (e.g., resveratrol with the deacetylase SIRT1[3]). Additionally, we and others have shown that some of the fluorescently labeled KDAC substrates are not active substrates when the fluorophore is absent.[8,16] We also note that the FdL assay uses trypsin and compounds that inhibit this protease, but not the KDAC, would represent false-positive hits. We therefore tested whether the six unconfirmed hits inhibit trypsin activity using a deacetylated FdL substrate, and we found that they did not, suggesting that the six compounds are false-positive hits that may result from an artifact of the fluorescent assay.

To compare the robustness of the SAMDI and FdL assays, we calculated the $Z'$-factor, which measures the ability of an assay to distinguish positive signals from background.[17] A value of 1.0 represents an ideal assay, though values greater than 0.7 are well-suited for high-throughput screening applications. For our

comparison, we ran one 384-well plate for SAMDI and one manufacturer provided 96-well plate for FdL, where half of the wells contained 1 $\mu$M KDAC8 (positive controls) and the remaining half were without enzyme (negative controls). We determined the mean and standard deviation for each assay and used them to calculate the $Z'$-factor. For SAMDI, we obtained a $Z'$-factor of 0.95, whereas we obtained a $Z'$-factor of 0.75 for FdL (Figure 3c). While the FdL $Z'$-factor value is acceptable as a high-throughput assay, SAMDI clearly shows a superior $Z'$-factor and, therefore, has much less potential for false-positive signals, as observed above. We also calculated the $Z'$-factor across the entire SAMDI screen and obtained a value of 0.84 (Figure 3b).

The SAMDI method also represents an advance over other important MS methods developed for screening applications. The desorption/ionization on silicon (DIOS) method, for example, has the primary benefit that it does not require matrix and can therefore analyze small molecules. Most applications of the DIOS method have not used covalent capture of the analyte and therefore can require more complex protocols for sample preparation. However, Finn, Siuzdak, and co-workers have reported a related approach that employs cleavable linkers and may offer another route to high-throughput applications.[18,19] Additionally, the RapidFire method allows high-throughput analysis using mass spectrometry but requires a solid phase extraction or chromatographic sample preparation of the analyte before mass analysis.[4] With SAMDI, the array plates (384 spots) need only be rinsed and treated with matrix after analyte immobilization.

This paper describes the use of SAMDI for high-throughput inhibitor screening. By translating the assay to 384-spot array plates and by accessing laboratory automation equipment and protocols, we show that the assay can screen a 100 000 molecule library in one day, making this method competitive with many mature technologies used in HTS.

## ■ ASSOCIATED CONTENT

**ⓢ Supporting Information.** Detailed experimental methods and supplemental figures describing inhibitor ranking and assay comparison. This material is available free of charge via the Internet at http://pubs.acs.org.

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