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Communication

High Throughput Screening with SAMDI Mass Spectrometry for Directed Evolution

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ABSTRACT: Advances in directed evolution have led to an exploration of new and important chemical transformations; however, many of these efforts still rely on the use of low-throughput chromatography-based screening methods. We present a high-throughput strategy for screening libraries of enzyme variants for improved activity. Unpurified reaction products are immobilized to a self-assembled monolayer and analyzed by mass spectrometry, allowing for direct evaluation of thousands of variants in under an hour. The method was demonstrated with libraries of randomly mutated cytochrome P411 variants to identify improved catalysts for C−H alkylation. The technique may be tailored to evolve enzymatic activity for a variety of transformations where higher throughput is needed.

Directed evolution represents a viable route to developing biocatalysts for synthetic organic chemistry,[1−4] including many non-natural transformations.[5−10] With substantial advances in our ability to generate genetic diversity[11] and prepare libraries exceeding thousands of variants,[12] the screening of activity remains a significant bottleneck for many reactions. Conventional screening efforts largely rely on optical methods, which oftentimes have a defined reaction scope[13,14] as they usually require a suitable chromophore on the molecule of interest[15−17] or are based on the detection of a coupled coproduct.[18] While methods employing mass spectrometry (MS) have the advantage that they are label-free and therefore quite general, a chromatographic separation step is often necessary, which can limit throughput.[12,19−22] These methods limit both the rate at which evolution is performed and the sequence space explored, often with only several hundred variants screened per round of evolution in a suitable time frame.[23,24] This constraint has prompted the development of alternative MS-based assays.[16,25−29] Here, we describe the first application of self-assembled monolayers for matrix-assisted laser desorption ionization (SAMDI) as a high-throughput assay that provides a generalizable platform to enable screening in directed evolution campaigns.

In recent work, we evolved a cytochrome P411 to perform alkylation of sp^3 C−H bonds through carbene C−H insertion, providing an efficient biocatalytic route for this highly challenging and valuable transformation.[12] However, each of the reactions reported required the use of chromatography to detect the reaction products. Leveraging SAMDI's abilities to assay enzyme activity[30,31] and rapidly analyze thousands of small molecule reactions directly from complex solutions,[32,33] we sought to continue evolving this catalyst for C−H insertion activity in high throughput. We chose an allylic substrate which was among the most challenging to detect[12] (Figure 1A); developing a screen for this reaction is difficult because the products are not easily ionizable, do not possess a significant chromophore or generate a fluorescent signal, and cannot be linked to the viability of the cell, coupled to a measurable coproduct, or make use of a biological reporter system.

To evolve enzymes for this reaction, we generate libraries containing cytochrome P411 variants in well plates and allow the variants to catalyze the reaction on an acetate-protected substrate (Figure 1A). With our approach, we then use self-assembled monolayers to selectively immobilize the substrate and reaction product directly from cell suspensions (Figure 1B). Based on the chemistry available on the reaction products, we chose to engineer the surface to present maleimide groups against a background of tri(ethylene glycol) groups. We can treat the reaction products with acid to reveal the thiol, which allows immobilization to the monolayer via a Michael addition. We then use SAMDI MS to measure the masses of the analyte-alkanethiolate conjugates by matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS).[34,35] In this way, we need only identify the products by a corresponding change in mass and integrate the peaks of the substrate and product to provide a yield for the reaction (Figure 1C).

In this study, the peak corresponding to substrate capture was present at 1033 Da, and the product peak was shifted by +86 Da (Figure 1C, right). For each spectrum acquired, we calculated relative product yields from the area under the curve (AUC) of each peak using $AUC_{product}/(AUC_{substrate} + AUC_{product})$. Each variant was screened in quadruplicate to acquire an average yield and account for variability in the deprotection and immobilization steps. We then normalized the values by the average value of parent activity on each respective plate to acquire a fold improvement. For each

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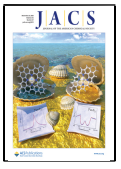

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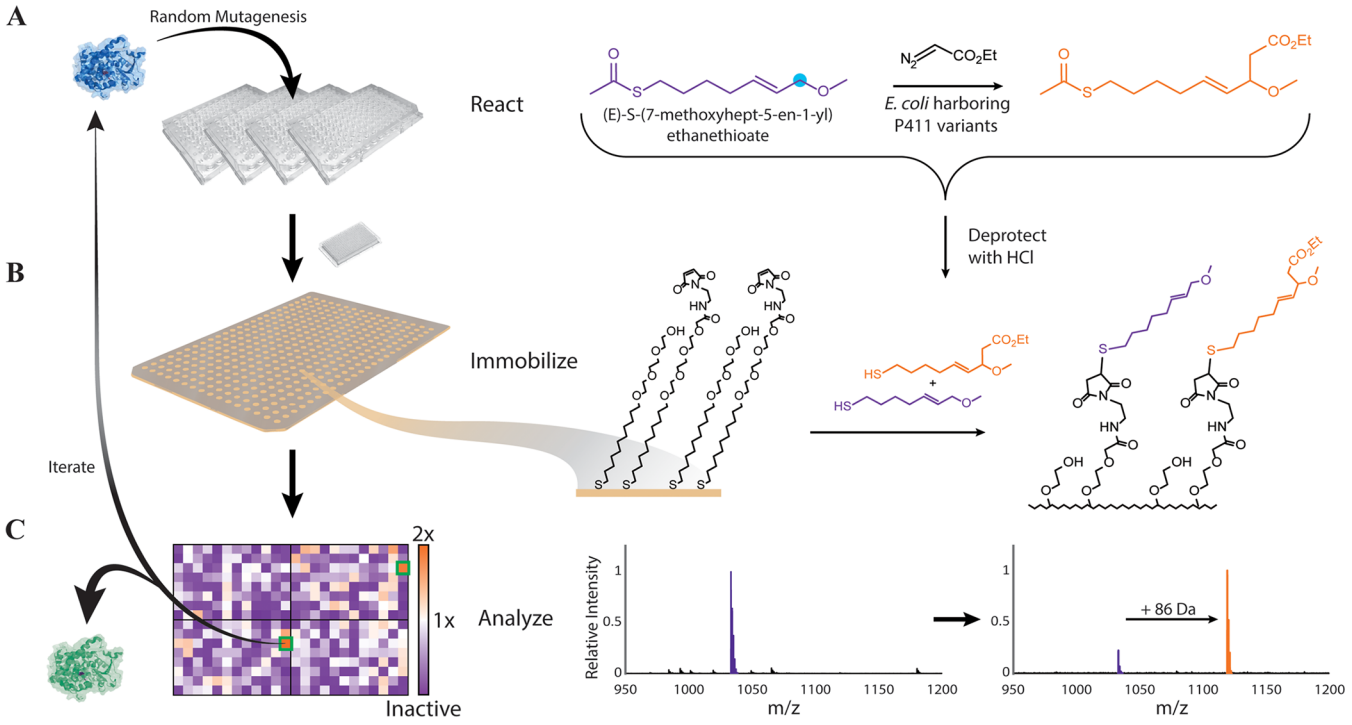

Figure 1. Use of the SAMDI screening assay in a cycle of directed evolution. (A) Libraries of cytochrome P411 are expressed in 96-well plates and allowed to react with the substrate (purple) and ethyl diazoacetate to form the ester product (orange). (B) Reaction products are deprotected and transferred directly to a SAMDI plate where they immobilize to a maleimide-presenting monolayer. (C) The array is analyzed by MS, and results are displayed as a heat map where each variant is shaded by fold improvement measured over its parent.

library, we generated heat maps to visualize the relative activities of each variant (Figure 1C, left). Variants were shaded by their fold improvement where orange represents increased activity and purple, decreased activity relative to parent-like activity (white). The most promising library members were run at analytical scale and validated using gas chromatography mass spectrometry (GCMS). We then selected the best variant to be the parent of the next round of evolution.

To identify the best starting variant for the first round of evolution, we screened a diverse panel of heme proteins (see SI methods) and chose a variant with one mutation (P74T) from P411-CHF identified by Zhang et al.[12] With P411-CHF-(P74T) as the initial parent, we first used SAMDI to measure the retention of function of enzymes in libraries generated by error-prone PCR at various manganese chloride concentrations (Figure S1). From these data, we found that SAMDI was able to rank variants with a least-squares correlation of $R^2 = 0.96$ to data collected by GCMS (Figure 2). While both techniques identified one variant in this library as having potentially improved activity, further validation confirmed this hit to be a false positive (Figure S2).

In order to identify biocatalysts with increased activity, we performed iterative rounds of mutagenesis and screening in whole *E. coli* cells. Because SAMDI can handle the large sequence space of random libraries, we opted to generate mutations throughout the entire gene using error-prone PCR. Over the course of three rounds of evolution, we acquired data for nearly 5000 variants (Figure S3) approximately 140-fold more rapidly than what would be expected with GCMS (Table 1). Here, data generation for each round required only a few hours, reducing the total analysis time from 24 days (for 1 replicate) to 17 h (for 4 replicates). In the third round we screened nearly 2500 variants and did not find a significantly improved enzyme, suggesting that the enzyme may be

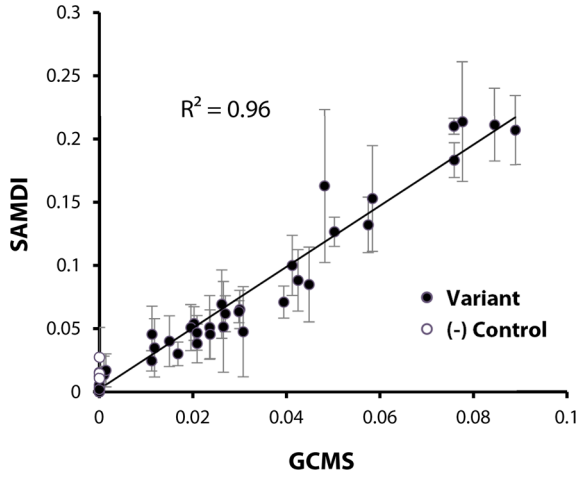

Figure 2. Scatterplot of screening results collected via GCMS and SAMDI from a library of 70 mutants. Values were calculated as a fraction of product over the total of the remaining starting material and product formed. Correlation was determined using least-squares linear regression.

Table 1. Comparison of Throughput and Total Screening Effort of SAMDI to Conventional Screening Methodology

	round 1	round 2	round 3	total
# of variants	540	1920	2496	4956
time GCMS 7 min/sample	63 h	224 h	291 h	24 days
time SAMDI 3 s/sample	27 min	96 min	125 min	248 min
x4 replicates	1.8 h	6.4 h	8.3 h	~17 h

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approaching a local maximum in activity or may need stabilizing mutations before further activating mutations can be found. Experimental procedure may also need to be reworked to avoid possible limitations in dynamic range as enzymes improve. As we sought to evolve on this platform as a proof of principle and managed to do so, we decided to end the campaign.

To acquire the fold improvement of each parent over their predecessor, we ran the top variants from each round at analytical scale and measured their activities against their parents by GCMS. The final variant displayed a 2-fold improvement (1300 total turnovers (TTNs)) (Figure 3).

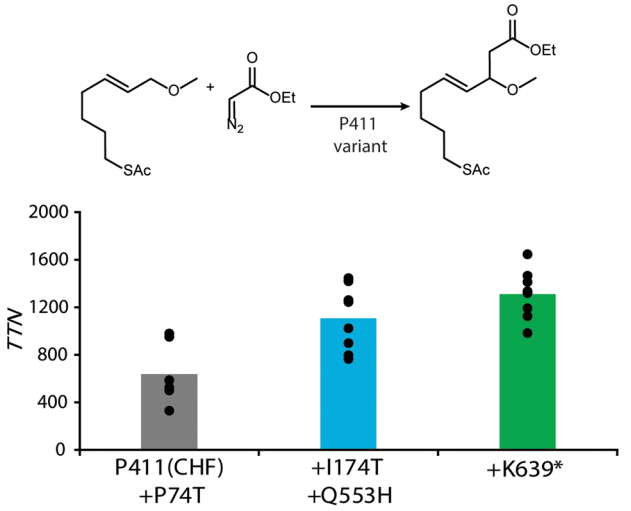

Figure 3. We used GCMS to characterize the hits and obtain the total turnover (TTN) for each variant. The evolutionary lineage of P411 for C−H alkylation is displayed. Bars represent mean yields (performed from two independent cell cultures, each used for duplicate reactions). Reaction conditions were as follows: cytochrome P411 in *E. coli* whole cells (optical density at 600 nm, OD600, of 1), 5 mM substrate, 5 mM ethyl diazoacetate, 5 vol % EtOH in M9-N buffer at room temperature under anaerobic conditions for 18 h. The asterisk symbol represents the introduction of a stop codon. See the SI for details.

Interestingly, none of the beneficial mutations were in the active site of the enzyme or at sites previously mutated in rational approaches (Figure S4). By not restricting the sequence space explored, we were able to identify potential allosteric effects and provide new sites that may be investigated in targeted evolution.

To demonstrate the reproducibility of the SAMDI technique, we selected and scaled up one variant from the final round to be screened repeatedly with SAMDI. Here we found a standard deviation of 2.3% with a resolving power of 0.1 m/z (Figure S5). The primary source of variability about the mean is likely due to application of matrix, which leads to modest differences in signal strength from spot to spot.[34] We also note that while the SAMDI technique is able to accurately quantitate the extent of each reaction,[36] we only required relative product yields to proceed with evolution, and thus, accurate yields were determined only for the variants validated by GCMS. Experimental reproducibility at-large was shown by inducing multiple colonies of the same clone for the initial variant. Here, we found a coefficient of variation (CV) of 14% (Figure S6).

The use of a thiol-tagged reactant to allow immobilization of the product was a convenient choice in this work but would not be compatible with many reactions. Hence, we repeated the C−H insertion reactions using a molecule that lacked a thiol group and that could be immobilized to a monolayer using a "traceless" immobilization method (Figure S7). In this scheme, a monolayer presenting a diazirine group reveals a carbene on irradiation, which reacts nonselectively to immobilize nearly all molecules.[37,38] We performed reactions for five additional substrates and used the traceless immobilization scheme to analyze products and, in each case, observed peaks in the mass spectra that corresponded to reactant and product (Figure S8). This example demonstrates that reactants need not be functionalized for immobilization and, in turn, suggests that this method will have a very broad relevance in directed evolution.

Throughout the course of this study, 22 944 spectra were generated and processed. With each plate requiring only 30 min per run, SAMDI collected data more than 100-fold faster than classic GCMS and approximately 10-fold faster than recent developments in the state-of-the-art thereof.[20,39] If higher throughput is desired, the method may be accelerated by approximately 2-fold by working with groups of 16 96-well plates in 50 min per run, with each sample requiring only 0.5 μL from each well.[33]

SAMDI-MS has been used extensively to profile enzymatic activity both in biochemical reactions and from complex lysates, while permitting the analysis of up to thousands of samples per hour and more than 30 000 experiments per day.[31,32,36,40−42] Hence, it is clear that the throughput in this study was not limited by the number of variants that could be screened.

The approach described here has the benefits that it is high-throughput, compatible with all library diversification techniques performed in multiwell plates and may be applied to any reaction that produces a shift in mass.[33] While epPCR allowed SAMDI to find modest fold improvements in the present work, we expect that utilizing other diversification techniques will result in greater improvements.

SAMDI-MS can accommodate a wide variety of chemical transformations—without sacrificing throughput—as other immobilization strategies have been demonstrated and are readily available.[35,37,38,40,43] In this way, the assay is not limited to certain classes of reactions but can be adapted to many organic transformations. We note that this method cannot be applied to reactions where the product and substrate share the same mass—including stereoisomeric and tautomeric structures—and would in those cases require a second reaction step (that is selective for one of the molecules), tandem mass spectrometry, or a separation step.

As directed evolution continues to add new chemistries to Nature's repertoire, generating small molecules with increasing complexity,[44] the need for high-throughput and generalizable screening tools is paramount. It is the use of immobilization chemistry that distinguishes SAMDI's throughput and substantiates the method to be well suited for evaluating variants in applications of directed evolution. This platform enables directed evolution efforts to evolve enzymes for improved activity and interrogate wider areas of protein space. We anticipate that further use of this method will lead to exploring larger areas of chemical space in high throughput and help uncover unexpected solutions for creating better enzymes.

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■ ASSOCIATED CONTENT

Supporting Information

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Additional figures and experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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