Applications of Mass Spectrometry in Combinatorial Chemistry

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Abstract: This article describes the use of two mass spectrometric techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry, toward a variety of challenging problems in drug discovery and identification. Quantitative ESI was used to screen for inhibitor activity of two different enzymatic glycosylation reactions resulting in the identification of the most effective inhibitors and the determination of their IC_{50} (inhibitor concentration at 50% inhibition). Also described is a combinatorial extraction method used with automated MALDI mass spectrometry to improve upon the clinical analysis of the immunosuppressant drug cyclosporin A (CsA). Optimization was performed by generating an array of solvent systems which were screened (by MALDI-MS) for the most efficient extraction of CsA from whole blood. Ultimately a 70/30 hexane:$CHCl_3$ mixture was identified as the most efficient binary solvent system for such extractions. In addition it was demonstrated that peptides and carbohydrates, covalently linked to a polymeric support (through a photolabile linker), can be directly analyzed by MALDI in a single step which requires no pretreatment of the sample to induce cleavage from the support. The UV laser light in the MALDI experiment was used to simultaneously promote the analyte's photolytic cleavage from the solid support and its gas phase ionization for subsequent mass spectral analysis. Overall, the strength of mass spectrometry lies in its versatility, making it a powerful analytical technique with which to characterize the diversity of compounds found in combinatorial libraries. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng (Comb Chem)* **61**:127–134, 1998.

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MASS SPECTROMETRY OVERVIEW

Mass spectrometry is playing an increasingly important role in the molecular characterization of combinatorial libraries, natural products, and biopolymers.[1–4] The development of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) has significantly extended the application of mass spectrometry toward a wide variety of challenging problems, including drug discovery, the identification of effective ligand-receptor binding, new catalysts, and enzyme inhibitors. Crucial to distinguishing the most active component or obtaining structure-activity relationships of compounds in a library is an efficient qualitative and quantitative assay. Toward this end, ESI and MALDI have been useful for the qualitative,[5–7] and more recently, the quantitative screening of combinatorial libraries.[8] In addition, mass spectrometry does not involve chromophores or radiolabeling and thus provides a viable alternative to existing analytical techniques, which typically require extensive sample preparation and optimization time, the disposal of biohazardous waste, and a significant amount of sample.

The utility of ESI lies in its ability to generate ions directly from the solution phase into the gas phase. The ions are created by applying a strong electric field to a very fine spray of the solution containing the analyte. The electric field creates highly charged droplets whose subsequent vaporization (or desolvation) results in the production of gaseous ions. The fact that ions are formed from solution has established the tech-

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nique as a convenient mass detector for liquid chromatography and for automated sample analysis. In addition, ESI-MS offers many advantages over other mass spectrometric methods, including the ability to analyze low mass compounds, excellent quantitation and reproducibility, high sensitivity, simple sample preparation, amenability to automation, soft ionization, and the absence of matrix.[9–12] In MALDI-MS, gas-phase ions are generated by the laser vaporization of a solid matrix/analyte mixture. The matrix (usually a small crystalline organic compound) is necessary to absorb the laser radiation, in effect acting as a receptacle for energy deposition. This concentrated energy deposition results in the vaporization and ionization of both matrix and analyte ions. In the following studies we have made use of the unique ionization qualities of both techniques for automation and quantitative analysis in combinatorial chemistry.

Automated Quantitative ESI-MS as an Assay for Enzyme Inhibitors

Using the advantages of ESI-MS detailed above we have investigated inhibitor libraries designed to inhibit enzymatic glycosylation reactions. In this approach, the entire enzymatic mixture (substrate, inhibitor, product, and internal standard) is introduced into the ESI mass spectrometer while analyzing for product formation as a function of the presence of inhibitor. Because only the product formation is quantitatively monitored, the effectiveness of the inhibitor can be readily determined.

We have applied this quantitative ESI-MS approach (Fig 1) to galactosyltransferase and fucosidase enzymes. Both enzymes are responsible for processing cell-surface carbohydrates, which in turn are associated with many specific recognition and signaling processes leading to important biological functions and disease.[13,14,8] Therefore, developing new assays and finding new inhibitors for glycoprocessing reactions is both medically relevant as well as scientifically interesting. To date, however, only limited success has been achieved in the design of these inhibitors. For the quantitation of glycosyltransferase activity, the current assays require the separation of the compounds, the use of radiolabeled sugars, the performance of large-scale reactions, or the use of additional enzymes.[15,16] Furthermore, radiolabeling and spectrophotometric assays are not suitable for the rapid screening of large libraries.

In the initial set of experiments, a galactosyltransferase-catalyzed reaction was examined in the presence of potential inhibitors.[8] The initial inhibitor

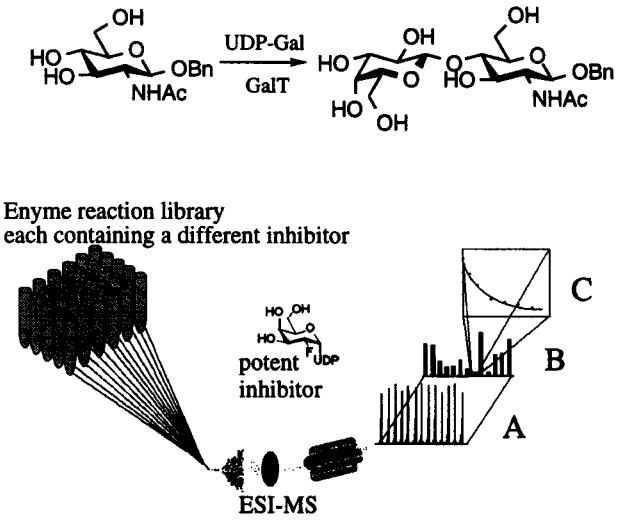

Figure 1. Quantitative determination of galactosyltransferase inhibition. (top) The enzyme reaction was monitored using an automated electrospray ionization mass spectrometry screening experiment. (bottom) Each solution contains the enzyme, reactants, an inhibitor (inset), and an internal standard. (A) The total ion current is recorded on each injection into the mass spectrometer. (B) Product formation is monitored with respect to the internal standard during each injection. (C) If an inhibitor is found to be effective, IC_{50} data can be generated using the ESI-MS.

library contained 20 inhibitor candidates.[14] The 20 candidates and 2 control reactions were assayed individually in 22 parallel reactions carried out and then quenched by adding MeOH (followed by the addition of an internal standard). The reaction mixtures were directly injected into the electrospray mass spectrometer with an autosampler. Three compounds, uridine-5′-diphosphate (UDP), uridine-5′-diphospho-(2-deoxy-2-fluoro)galactose (UDP-2F-Gal) and uridine-5′-triphosphate (UTP) were found to be the most potent inhibitors of the galactosyltransferase (Figure 2). Additional libraries were generated in which the concentration of the inhibitors were varied to determine the IC_{50} (inhibitor concentration at 50% inhibition) of UTP, UDP and UDP-2F-Gal. The mass spectral results showed the IC_{50} to be 600 μM for UTP, 200 μM for UDP and 120 μM for UDP-2F-Gal. UDP-2F-Gal was thus identified as the best inhibitor. This strategy is also applicable to the screening of glycosidase inhibitors.

In a representative case, using *p*-nitrophenyl-α-L-fucoside as the substrate, the rate of fucosidase catalyzed hydrolysis was measured by monitoring the formation of *p*-nitrophenol in the presence of inhibitors. The result of these experiments allowed for the identification of an inhibitor with nanomolar IC_{50}.[14]

In an effort to further increase the screening capacity, multiple inhibitors were screened against one enzyme simultaneously. The experiments were per-

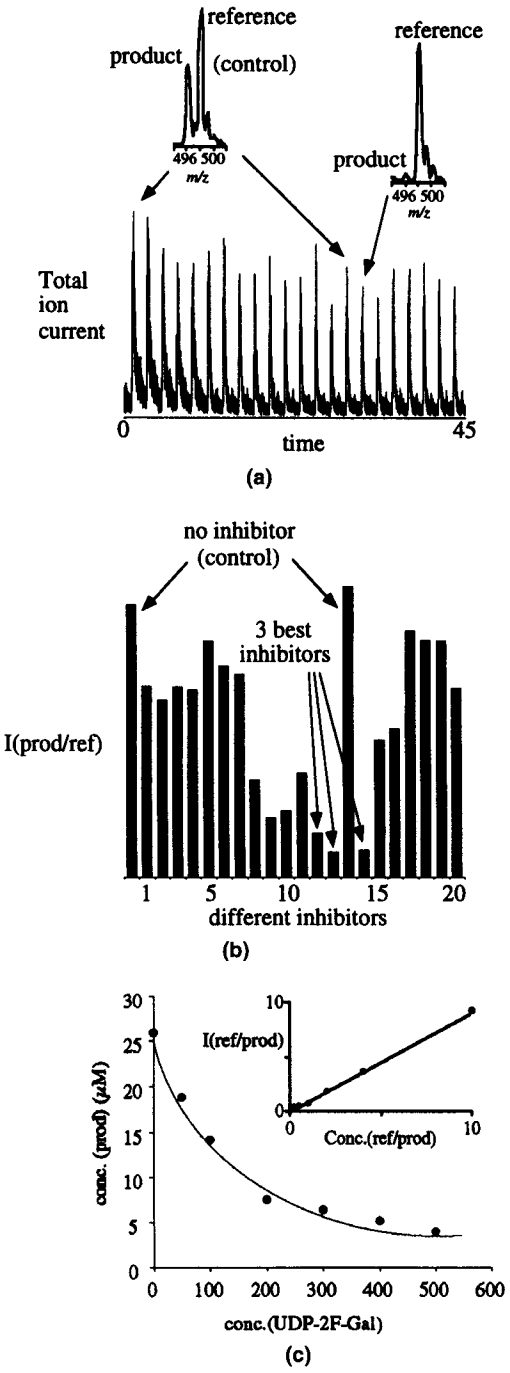

Figure 2. (A) The electrospray ionization total ion current observed for the 22 reactions (each having a different inhibitor). Inserts are the mass spectra for the reactions with no inhibitor (left, top) and an inhibitor found to be effective (right, top). (B) The bargraph of product ion/reference ion intensity ratio generated from the mass spectrum of each reaction containing potential inhibitors. (C) The product of the galactosyltransferase catalyzed enzyme reaction was also monitored as a function of inhibitor concentration to generate the IC_{50} data.[8]

formed by pooling five potential inhibitors and the enzyme into one reaction vessel. The reactions that contained five inactive compounds showed no inhibition, whereas the reactions containing four inactive compounds and one active inhibitor (UDP) showed potent inhibition. The compounds in the inhibited reaction were then individually analyzed to identify the actual inhibitor compound. Such strategies increased the screening capacity several fold such that one inhibitor/2 min allowed for 720 inhibitors/d, while five inhibitors/2 min allowed for 3600 inhibitors/d.

While we have shown the effectiveness of this method in the screening of inhibitors for enzymes involved in carbohydrate processing, one can envision how this approach can be extended toward screening libraries of other classes of enzymes (such as enzymes with peptide-based substrates) or for screening metal-catalyzed chemical reactions. Indeed, this method can be applied toward screening any reaction whose product molecule is amenable to mass spectrometric analysis.

In the previous example, ESI-MS was the mass spectrometric method of choice mainly because of its ability to analyze low mass compounds and its amenability to the direct injection of the reaction mixture. As the following section will show, MALDI-MS, although requiring the use of a matrix, offers many of the same advantages (automation, soft ionization, and excellent quantitation) and in addition, is able to analyze high mass compounds and relatively heterogeneous mixtures.

Automated Quantitation Studies Using MALDI-MS

One of MALDI's greatest attributes is its ability to analyze complex heterogeneous mixtures. This ability has made MALDI a common and valuable technique in the analysis of biological fluids,[17] analyses commonly performed by immunoassays and high-performance liquid chromatography (HPLC). Immunoassays, however, generally have low reproducibility and reliability, and provide little to no selectivity between a drug and its metabolites.[18,19] This lack of specificity is a significant limitation because metabolites, although structurally similar to the parent compound, often have different biological activity.[20,21] Mass spectrometric analysis, on the other hand, allows for co-extracted metabolites to be identified and quantitatively monitored (unless they have the same molecular weight). Although HPLC is relatively selective and accurate, the sensitivity is very compound-dependent and method development can be time-consuming. Thus, while both techniques are useful, both suffer when compared to the speed, sensitivity, and accuracy offered by mass spectrometry.[19,22,23]

Because some biofluids contain contaminants, it is often necessary to perform extractions for purification

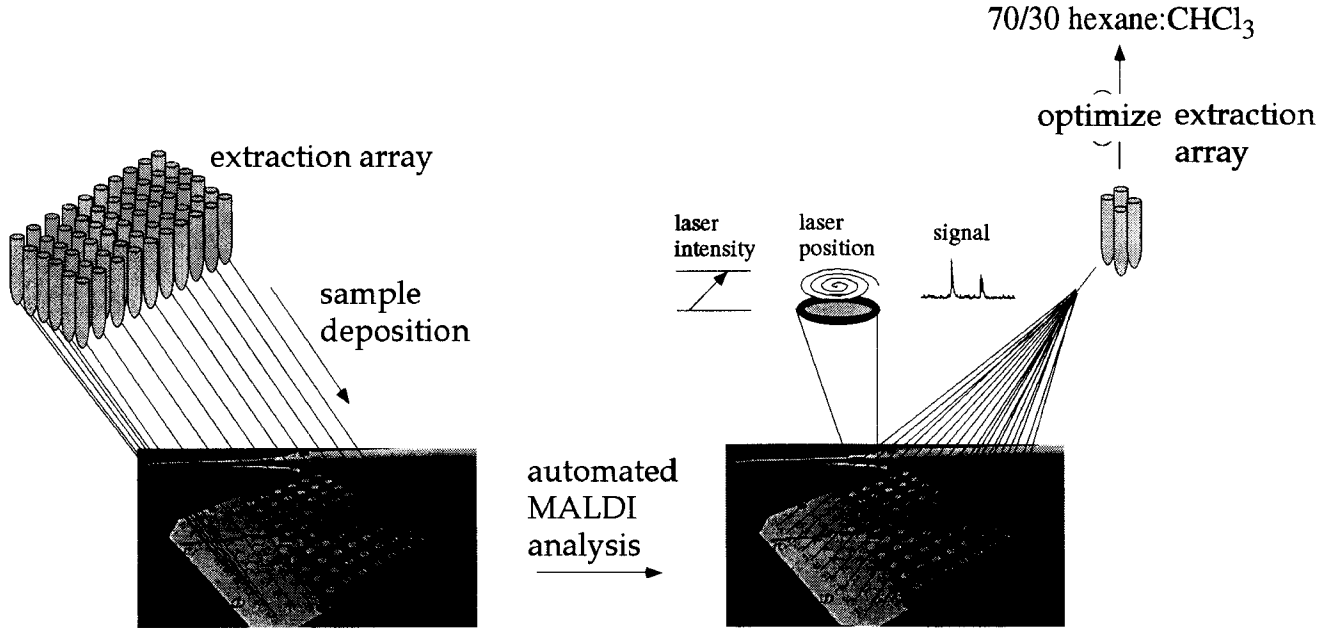

Figure 3. The automated MALDI mass spectrometry experiments were performed as a function of laser position (within each well) and laser intensity for each extracted CsA sample. The first iteration of these extraction experiments generated four useful solvent systems, and the second generation produced an effective 70/30 hexane:$CHCl_3$ extraction solvent system.

prior to sample analysis. In such instances the development of an efficient extraction assay can be very time-consuming. In an effort to create a more simplified and efficient extraction protocol, we have developed a combinatorial extraction method to be used with an automated MALDI mass spectrometry procedure.[23] In this example we demonstrate the approach to improve clinical analysis of the immunosuppressant drug cyclosporin A (CsA). Figure 3 shows the combinatorial-extraction approach followed by MS analysis, in which a MALDI mass spectrometer equipped with automated multi-sampling capabilities was used to facilitate data collection and analysis of cyclosporin A. The organic layer extracted from blood for each sample was placed on a MALDI sample plate (with a capacity for 100 samples) and was then loaded into the mass spectrometer and analyzed using a computer-controlled algorithm. Extraction optimization was performed by generating an array of solvent systems and an automated analysis to identify successful extractions. The first generation of experiments revealed four effective binary solvent systems (hexane/EtOH, ACN/H_2O, ACN/MeOH, hexane/$CHCl_3$). A new array based on these solvent systems was generated and in a second iteration of these experiments, hexane/$CHCl_3$ (70:30) was found to provide the most effective single-step extraction for cyclosporin and its metabolites (Figure 4). The limits of detection were determined to be 15 ng/mL in whole blood for both ESI/MS and MALDI-MS and could be used to identify major drug metabolites. The 70/30 hexane/$CHCl_3$ extraction

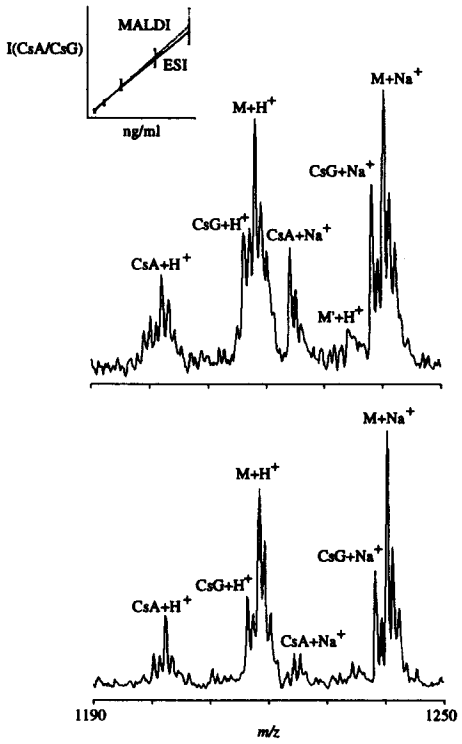

Figure 4. (inset) Calibration curves generated using MALDI and ESI for the quantitation of CsA. (top) MALDI spectrum of a patient blood extract containing CsA spiked with CsG. The extraction procedure was performed using the traditional multistep method. (below) MALDI spectrum of a patient blood extract containing CsA spiked with CsG using single-step extraction procedure developed with the combinatorial approach. M (M8, M17 and/or M18) and M′ (M10 and/or M16) represent different metabolites of CsA that have the same mass.

also proved to be a much faster method, typically requiring 5 minutes to perform.

To determine the efficiency of our new extraction procedure, it was compared to the previously developed "ether" extraction.[24] Initially, calibration curves were obtained using the "ether" extraction (a plot of the ratio of Σint.[$CsAH^+$ + $CsANa^+$]/[$CsGH^+$ + $CsGNa^+$] vs. CsA concentration) with both the ESI and MALDI mass spectrometers (cyclosporin G, CsG, was used as the internal standard). The data exhibited a linear relationship in the concentration range (0 to 1500 ng/mL) (Table I) with an excellent correlation coefficient (R = 0.999) for both electrospray and MALDI. The new extraction generated standard curves for CsA that were very similar to those from the "ether" extraction method. Figure 4 shows the calibration curves generated using MALDI and ESI for the quantitation of CsA and the MALDI spectra of whole blood extract CsA.

Both approaches described above, screening for enzyme inhibitor activity and optimizing extraction conditions, represent only a few of the many ways in which mass spectrometry can be used in the field of combinatorial chemistry. In addition to the development of new quantitative methodologies, the qualitative evaluation of combinatorial libraries is essential for the characterization of active compounds and for monitoring both the progression and efficiency of reaction pathways. Because many libraries are synthesized on solid polymeric supports (e.g., resin beads), this chemistry necessitates assays which allow for characterization directly from the solid support. In the following section we utilize both MALDI-MS and photolabile linkers as a means of directly characterizing compounds from the solid phase.

Characterizing Compounds and Reactions Directly from the Solid Phase

Several reports have shown the utility of mass spectrometry in the characterization of compounds subsequent to their chemical cleavage from solid polymeric supports.[25–29] Demonstrated here is a new MALDI approach which allows compounds covalently bound to a single polymeric bead (~50 μm in diameter) or set of beads to be mass analyzed prior to their chemical cleavage from the resin.[30] The scheme, outlined in Figure 5, permits the characterization of resin-bound analytes in a single step, which requires no pretreatment of the sample to induce cleavage from the support. We have demonstrated that peptides covalently linked to a polymeric support through a photolabile linker can be directly analyzed by MALDI-MS. In addition, we also show that the technique is suitable for monitoring chemical reactions on the solid phase.

The peptide KPAFLKPQFLG was manually synthesized from protected amino acids in a stepwise fashion using in situ neutralization/HBTU activation protocols for Boc chemistry.[31] The synthesis was initiated on Br-Wang resin (obtained from Novabiochem,

Table I. The results obtained from the ESI-MS and MALDI-MS analysis of extracted cyclosporin A from standard blood samples using the 70/30 hexane/$CHCl_3$ extraction.

Standard ng/mL	ESI ng/mL (error)	MALDI ng/mL (error)
100	98 (2%)	94 (6%)
250	231 (8%)	238 (5%)
500	523 (5%)	543 (9%)
1000	1009 (1%)	961 (4%)
1500	1404 (6%)	1514 (1%)

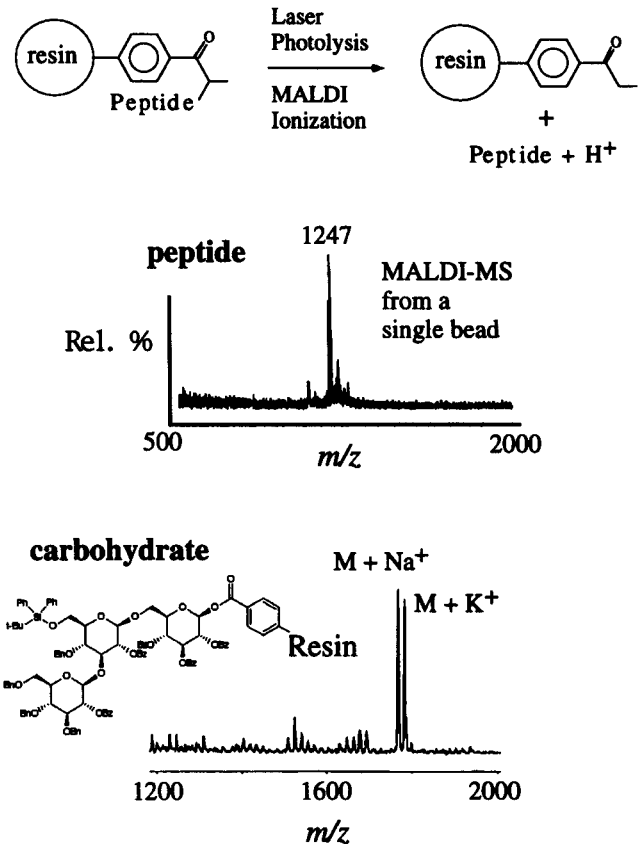

Figure 5. The MALDI technique facilitates the vaporization and ionization of analyte molecules by irradiating the sample/matrix mixture with a UV laser. (top) Schematic representation of the products generated upon laser photolysis (by MALDI) of a peptide covalently attached to a solid phase resin through a photolabile α-methylphenacyl-ester linker. (middle) The mass spectrum of the unprotected peptide KPAFLKPQFLG (MH^+ = 1246.5 Da) covalently attached to a polystyrene resin is a summary of 30 laser pulses acquired from a single bead. (below) The carbohydrate (courtesy of Nicolas Winssinger and K. C. Nicolaou) was also covalently linked to the resin through a photolabile linker. Data was obtained directly from the resin using sinapinic acid matrix.

San Diego, CA) that consists of a brominated α-methylphenacyl linker attached to a polystryrene-1% divinyl benzene support.[32,33] Prior to mass spectral analysis, resin samples were treated with 20% (v/v) piperdine in DMF in order to remove the Fmoc protecting groups, and then washed with 50/50 (v/v) dichloromethane/methanol. For MALDI analysis, ~1 mg of the deprotected peptide resin was added to 5 mL of ethanol. A 2 μL ethanol/bead suspension containing ~50 beads was then deposited on the MALDI sample plate, prior to the addition of 2 μL of a saturated ethanol solution of the matrix; α-cyano-4-hydroxycinnamic acid. It is important to note that our sample preparation involved the addition of 5 mL of ethanol to the resin-bound peptide. When the peptide resin was resuspended in smaller volumes (0.01–1.0 mL) of solvent, we detected free peptide in the MALDI analysis of the supernatent. Presumably, trace amounts of peptide (<1 nmol or <1% of the resin bound material) were present as a result of photolytic cleavage by ambient light. Our primary goal in this work was to determine if the MALDI experiment could be used to simultaneously promote both photolytic cleavage (from the resin bead) and gas-phase ionization of the analyte for subsequent mass spectral analysis. Therefore, it was important to suspend the peptide resin in a large solvent volume (~5 mL) to sufficiently dilute the residual free peptide in the sample to undetectable levels.

Figure 5 shows the mass spectrum of the unprotected, resin-bound peptide. MALDI analysis yielded a characteristic $[M + H]^+$ signal at *m/z* 1247 which is consistent with the expected mass of the free peptide (MW = 1245.5 Da). The spectrum in Figure 5 was acquired from over 30 laser pulses on a single bead. Mass spectra obtained from a single bead with one laser pulse (3 nanoseconds in duration) indicated that photolytic cleavage and ionization of material from the bead occurred during the laser pulse, as the resin sample was not exposed to laser light prior to analysis. The visualization system on the PerSeptive Voyager Elite instrument allowed irradiation of very specific regions of the sample plate; peptide signal was only observed when individual matrix/bead deposits were irradiated on the plate. No peptide signal was observed upon irradiation of matrix crystals not associated with resin beads, suggesting that there was little or no free peptide in the sample prior to MALDI analysis. These same observations were made even after repeated MALDI sample preparations on the same set of beads, indicating that there was no appreciable cleavage of the peptide from the resin during our MALDI sample preparation. Also as expected, in the absence of matrix the analysis of the resin-bound peptide produced no signal. Treatment of the resin (before and after MALDI analysis) with 20% (v/v) hydrazine in methanol gave the corresponding peptide hydrazide (MH^+ = 1261), as confirmed by MALDI and ESI mass spectrometry (data not shown). Our ability to recover the peptide (in a chemical cleavage step) from resin samples subjected to MALDI analysis indicates that the sample was not completely photolyzed in the experiment. This is not surprising, as only a small amount of material (<femtomoles) is typically consumed during such analysis.[34] Figure 5 also shows the MALDI analysis of resin-bound carbohydrate, illustrating the application of this technique to another class of compounds. Observed are ion signals representing the $[M + Na]^+$ the $[M + K]^+$ carbohydrate species.

The rapid, one-step MALDI procedure for the direct analysis of resin-bound molecules described above is ideally suited for studying chemical reactions on the solid phase. As a demonstration of the utility of MALDI in such an analysis, the coupling reaction of a Boc-Arg(Tos) residue (preactivated as the hydroxybenzotriazole ester) to the protected 11 amino acid peptide (described above) was monitored as a function of time. Interestingly, the Fmoc protected peptide was not amenable to MALDI analysis. The absence of appropriate protonation sites in this sample likely prevents ionization during the MALDI process. Therefore, prior to analysis resin samples were treated with 20% (v/v) piperdine in DMF to remove the Fmoc protecting groups. Resin samples taken at 0, 1, and 6 min time points were subjected to MALDI analysis, the results of which are shown in Figure 6. Our results show that the reaction proceeded quickly and coupling appeared nearly complete in just 6 minutes.

The direct analysis of resin-bound molecules by MALDI offers several important advantages. The first being the lack of an additional cleavage step prior to mass analysis, which is often required by other methods of characterization. Performing MALDI directly on the solid phase requires less sample handling and more efficient management because the resin-bound compound can be easily recovered for subsequent manipulations (again, less than femtomole quantities of material is consumed in typical MALDI analyses). The most significant advantage is that it can be used to monitor chemical reactions on the solid phase in real time, in much the same way that thin-layer chromatography is used to monitor reactions in solution. Furthermore, all analytes amenable to MALDI ionization

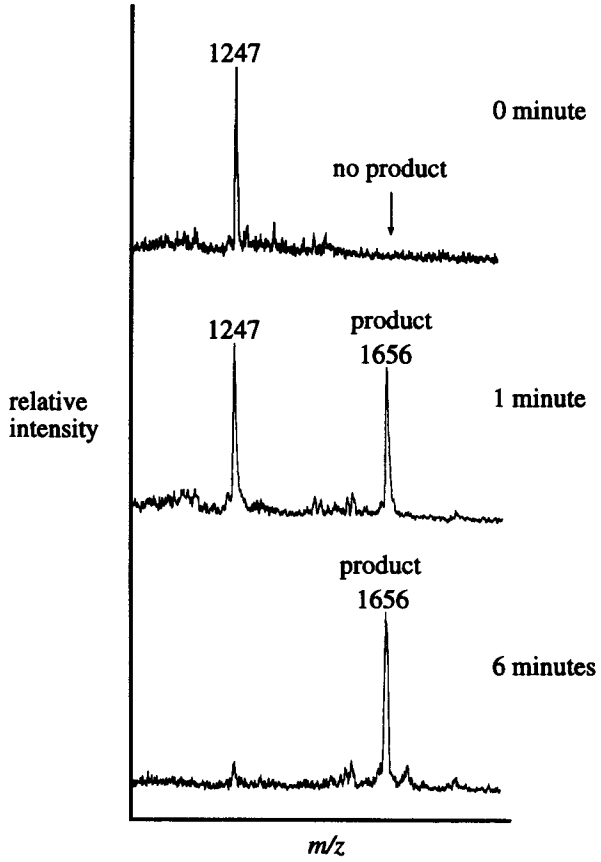

Figure 6. MALDI mass spectra showing the time course of reaction for the coupling of Boc-Arg(Tos) (MW = 428.5 Da) to the protected resin-bound peptide of sequence KPAFLKPQFLG. MALDI analysis was performed directly on peptide-resin samples after deprotection of the lysine side chain amino groups. Resin samples were taken after the reaction and allowed to proceed for 0, 1, and 6 minutes.

should prove suitable for routine analysis by this procedure.

CONCLUSION/SUMMARY

The rapid growth of combinatorial libraries has created a need for faster, more accurate, and more sensitive analytical techniques capable of large-scale (high-throughput) screening. Numerous improvements in speed, sensitivity and accuracy, together with innovations in both automation and quantitation place mass spectrometry among the most powerful analytical techniques available today. Quantitative ESI-MS was shown to be an effective assay for the identification of inhibitor activity in a combinatorial library. Potent nucleotide inhibitors of a galactosyltransferase were identified using this method. Clearly, this approach can be applied to a variety of different reaction systems. A combinatorial extraction method in combination with an automated MALDI mass spectrometric procedure was also used to optimize the clinical analysis of the immunosuppresant drug CsA from whole blood. In addition, MALDI was shown to be an effective method for the direct analysis of resin-bound compounds (without prior chemical cleavage from the resin), an ideal technique for the identification/characterization of combinatorial compounds synthesized on solid supports. Overall, the strength of mass spectrometry lies in such versatility, making it a powerful analytical technique with which to characterize the diversity of compounds found in combinatorial libraries.

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References

1. Cheng, X.; Chen, R.; Bruce, J. E.; Schwartz, B. L.; Anderson, G. A.; Hofstadler, S. A.; Gale, D. C.; Smith, R. D. *J. Am. Chem. Soc.* **1995**, *117*, 8859–8860.
2. Youngquist, R. S.; Fuentes, G. R.; Lacey, M. P.; Keough, T. *J. Am. Chem. Soc.* **1995**, *117*, 3900–3906.
3. Boutin, J. A.; Hennig, P.; Lambert, P. H.; Bertin, S.; Petit, L.; Mahieu, J.-P.; Serkiz, B.; Volland, J.-P.; J.-L., F. *Anal. Biochem.* **1996**, *234*, 126–141.
4. Gao, J.; Cheng, X.; Chen, R.; Sigal, G. B.; Bruce, J. E.; Schwartz, B. L.; Hofstadler, S. A.; Anderson, G. A.; Smith, R. D.; Whitesides, G. M. *J. Med. Chem.* **1996**, *39*, 1949–1955.
5. Boyce, R.; Li, G.; Nestler, H. P.; Suenaga, T.; Still, W. C. *J. Am. Chem. Soc.* **1994**, *116*, 7955–7956.
6. Liang, R.; Yan, L.; Loebach, J.; Ge, M.; Uozumi, Y.; Sekanina, K.; Horan, N.; Gildersleeve, J.; Thompaon, C.; Smith, A.; Biswas, K.; Still, W. C., Kahne, D. *Science* **1996**, *274*, 1996.
7. Georgiou, G.; Stathopoulos, C.; Daugherty, P. S.; Nayak, A. R., Iverson, B. L.; Curtiss III, R. *Nature Biotech.* **1997**, *15*, 29–34.
8. Wu, J.; Takayama, S.; Wong, C.-H.; Siuzdak, G. *Chem. Biol.*. **1997**, *4*, 653–657.
9. Lee, E. D.; Muck, W.; Henion, J. D.; Covey, T. R. *J. Am. Chem. Soc.* **1989**, *111*, 4600–4604.
10. Henion, J. D.; Nlordehai, A. V.; Cai, J. Y. *Anal. Chem.* **1994**, *66*, 2103–2109.
11. Hegy, G.; Gorlach, E.; Richmond, R.; Bitsch, F. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1894–1900.
12. Siuzdak, G. *Mass Spectrometry for Biotechnology;* Academic: San Diego, 1996.
13. Varki, A. *Glycobiol.* **1993**, *3*, 97–130.
14. Takayama, S.; Martin, R.; Wu, J.; Laslo, K.; Qiao, L.; Siuzdak, G.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 8146–8151.
15. Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Garcia-Junceda, E.; Williams, M. A.; Bayer, R.; Ketcham, C.; Walker, L. E.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 9283–9298.
16. Murray, B. W.; Wittmann, V.; Burkart, M. D.; Hung, S.-C.; Wong, C.-H. *Biochem.* **1997**, *36*, 823–831.
17. Rideout, D.; Bustamante, A.; Siuzdak, G. *Proc. Nat. Acad. Sciences USA.* **1993**, *90*, 10226–10229.
18. Rustum, A. M. *J. of Chromatogr. Sci.* **1990**, *28*, 594–598.
19. Muddiman, D. C.; Gusev, A. I.; Proctor, A.; Hercules, D. M.;

Venkataramanan, R.; Diven, W. *Anal. Chem.* **1994**, *66*, 2362–2368.
20. Rosano, T. G.; Freed, B. M.; Cerilli, J.; Lempert, N. *Transplantation.* **1986**, *42*, 262–267.
21. Freed, B. M.; Rosano, T. G.; Lempert, N. *Transplantation.* **1987**, *43*, 123–127.
22. Muddiman, D. C.; Gusev, A. I.; Langner, K. S.; Proctor, A.; Hercues, D. M.; Tata, P.; Venkataramanan, R.; Diven, W. *J. Mass Spec.* **1995**, *30*, 1469–1479.
23. Wu, J.; Chatman, K.; Harris, K.; Siuzdak, G. *Anal. Chem.* **1997**, *69*, 3767–3771.
24. Garraffo, R.; Lapalus, P. *J. Chromatogr.* **1985**, *337*, 416–422.
25. Brummel, C. L.; Lee, I. N.; Zhou, Y.; Benkovic, S. J.; Winograd, N. *Science* **1994**, *264*, 399.
26. Egner, B. J.; Cardno, M.; Bradley, M. J. *Chem. Soc.-Chem. Comm.* **1995**, *21*, 2163.
27. Egner, B. J.; Langley, G. J.; Bradley, M. *J. Org. Chem.* **1995**, *60*, 2652.
28. Haskins, N. J.; Hunter, D. J.; Organ, A. J.; Rahman, S.; Thom, C. *Rapid Comm. Mass Spectrom.* **1995**, *9*, 1437.
29. Brummel, C. L.; Vickerman, J. C.; Carr, S. A.; Hemling, M. E.; Roberts, G. D.; Johnson, W.; Weinstock, J.; Gaitonopoulos, D.; Benkovic, S. J.; Winograd, N. *Anal. Chem.* **1996**, *68*, 237.
30. Fitzgerald, M. C.; Harris, K.; Shevlin, C. G.; Siuzdak, G. *Biorg. Med. Chem. Lett.* **1996**, *6*, 979–982.
31. Schnölzer, M.; Alewood, P. A. J.; Alewood, D.; Kent, S. B. *Int. J. Pep. Prot. Res.* **1992**, *40*, 180.
32. Sheehan, J. C.; Umezawa, K. *J. Org. Chem.* **1973**, *38*, 3771.
33. Wang, S. S. *J. Org. Chem.* **1976**, *41*, 3258.
34. Cerpa-Poljak, A.; Jenkins, A.; Duncan, M. W. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 233–239.