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Evaluation of Mass Spectrometric Methods Applicable to the Direct Analysis of Non-Peptide Bead-Bound Combinatorial Libraries

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Electrospray, matrix-assisted laser desorption, and time-of-flight secondary ion mass spectrometry have been explored as possible methods for the identification of active members of molecular combinatorial libraries. All three methods are found to yield accurate molecular weight information about a target molecule angiotensin II antagonist synthesized on a 40-μm polystyrene bead. Structural identification is also possible by accurate mass measurements to eliminate candidate structures with the same nominal mass and by analysis of the fragmentation patterns. In addition, the secondary ion mass spectrometry measurements yield spatially resolved spectra from a single bead after exposure to a suitable gas which clips the covalent bond at the linking position. All three approaches appear to offer a viable screening strategy of non-peptide libraries without the use of additional molecular tags.

We report on the merits of three mass spectral techniques for the characterization of small organic molecules covalently attached to polystyrene beads. A rapid and straightforward method for this characterization is urgently needed due to the developing importance of combinatorial synthetic methods whereby a series of steps with multiple reagent choices for each step provides a large repertoire of compounds. Peptides have been most intensively investigated since synthetic methods are available to produce libraries with millions of members and since they are appropriate probes for receptor and enzyme function.[1,2] Ultimately it is expected that libraries of peptide mimics or other small organic molecules will yield viable drug candidates.[3]

The active compounds generated in bead-bound libraries are presently identified after release by a recursive analysis for a nontagging synthetic strategy or by direct analysis of an accompanying tag molecule from a positive bead.[4] Identification is achieved using traditional amplification and/or sequencing techniques for nucleotides and peptides or by electron capture, capillary gas chromatography if tag molecules are involved. Large libraries may contain several different biologically active compounds. If it is desired to test most of the compounds present in the library, then the statistics require that a relatively large sample of beads be assayed.[5] The result is that most of the compounds will be represented numerous times in the bioassay. Thus, a

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(5) In order to ensure that the majority of bead-bound compounds in a library are tested in the bioassay, the bead library must be oversampled. For example, Poisson statistics dictate that, for a bead library of 1000 compounds, 9000 beads must be sampled in order to ensure that every compound is present on at least one bead in the bioassay. In such a sample, the average number of beads on which a compound will be found is ~9, and some compounds will be found on as many as 19 different beads.

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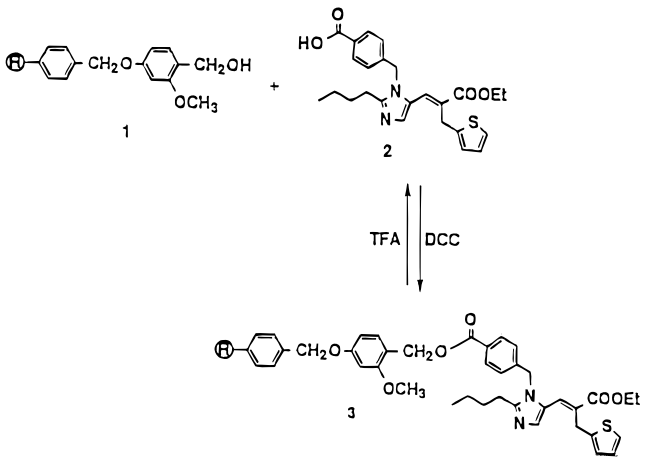
single library may contain many beads requiring structural analysis of the bound compounds. The appearance of more complex solid phase synthetic strategies, where heterocyclic networks may extend in three dimensions, further increases the burden on existing analysis strategies.

Mass spectral (MS) techniques have also been successfully employed to determine the structure of compounds that were synthesized on single beads without the use of separate tags.[6–9] In one case, a peptide released from the bead using a standard chemical cleaving agent was analyzed by matrix-assisted laser desorption ionization MS (MALDI-MS) to determine the molecular weight of the compound and any side products.[8] Peptide released from a bead has also been characterized by MALDI-MS for molecular weight and by analysis of the metastable fragment ions for structural information.[6] In what might be considered a self-tagging procedure, capped partial peptides generated as minor components during synthesis of peptides on beads were analyzed by MALDI-MS to yield sequence information from molecular weights of the partial peptides released from active beads.[7] In another set of experiments, the covalent bond between the peptide and the bead is severed with a gas phase reagent which leaves the molecule in place. Structural determination is then achieved using imaging time-of-flight secondary ion mass spectrometry (TOF-SIMS).[9] The imaging capability allows parallel analysis of multiple beads, each coated with a different peptide. These approaches offer an important alternative strategy to resolving the address problem in combinatorial libraries that does not involve coding or tagging methods.[10–12]

In this paper, we examine the merits of MS techniques for the direct identification of active small organic molecules which originate as members of generalized combinatorial libraries. Three approaches are examined in detail including electrospray MS (ESMS), MALDI-MS, and imaging TOF-SIMS using as a model angiotensin II (AII) receptor antagonist synthesized on bead. This compound was developed to block the renin–angiotensin system for the treatment of heart and chronic renal failure.[13] The results show that all three MS methods provide molecular weight and structural information with the requisite sensitivity needed for routine bead addressing. TOF-SIMS is shown to yield spatially resolved spectra directly from a single bead with mass accuracy of ~4 ppm for the molecular ion, but provides fewer structurally informative fragments. High mass accuracy is also available using ESMS, which reduces molecular weight redundancies and thereby reduces the number of candidate structures to a small subset of the library or even to a unique member. ESMS/MS and analysis of metastable ions in MALDI-MS also provide significant structural information that may be used to help distinguish between members of a library with the same elemental composition but different structures. These approaches are evaluated in terms of the impact they may eventually exert on the implementation of new screening strategies for generalized libraries.

EXPERIMENTAL METHODS

The carboxylic acid **2** was attached to Sasrin polystyrene beads **1** (Bachem AG,[12] average bead diameter 50 μm, 50 to 200 pmol of linker per bead) using dicyclohexylcarbodiimide (DCC) as the coupling agent. The acid **2** was prepared by the condensation of



2-butyl-1-[(4-carboxyphenyl)methyl]-1*H*-imidazole-5-carboxaldehyde and 2-carbethoxy-3-(2-thienyl)propanoic acid in a Knoevenagel reaction.[14]

For ESMS studies, a single bead containing the angiotensin II antagonist was placed in a micro-Eppendorf tube and exposed to a 1% solution of trifluoroacetic acid (TFA) in CH_2Cl_2 for 15 min. The resulting solution was dried and extracted into 10 μL of CH_3CN. An aliquot of ~1 μL of this solution was then introduced by flow injection electrospray into a Perkin-Elmer Sciex API-III triple–quadrupole mass spectrometer (Thornhill, ON, Canada). Tuning and calibration were carried out using a mixture of polypropylene glycols (PPGs) 425, 1000, and 2000. MS data were acquired at 10 data points per Da/e (0.1-Da step); MS/MS data were acquired at 5 data points per Da/e (0.2 Da step). Several scans (7–20) were averaged and the centers of mass determined for subsequent data analysis.

Once the molecular weight of the library member was determined, a second 1-μL aliquot of the CH_3CN solution was employed to obtain structural analysis via standard MS/MS procedures. For these experiments, the quasimolecular ion is selected by the first quadrupole lens and activated by collisions with Ar at 25 eV in the second quadrupole lens. The product ions are detected in the third analyzer.[15]

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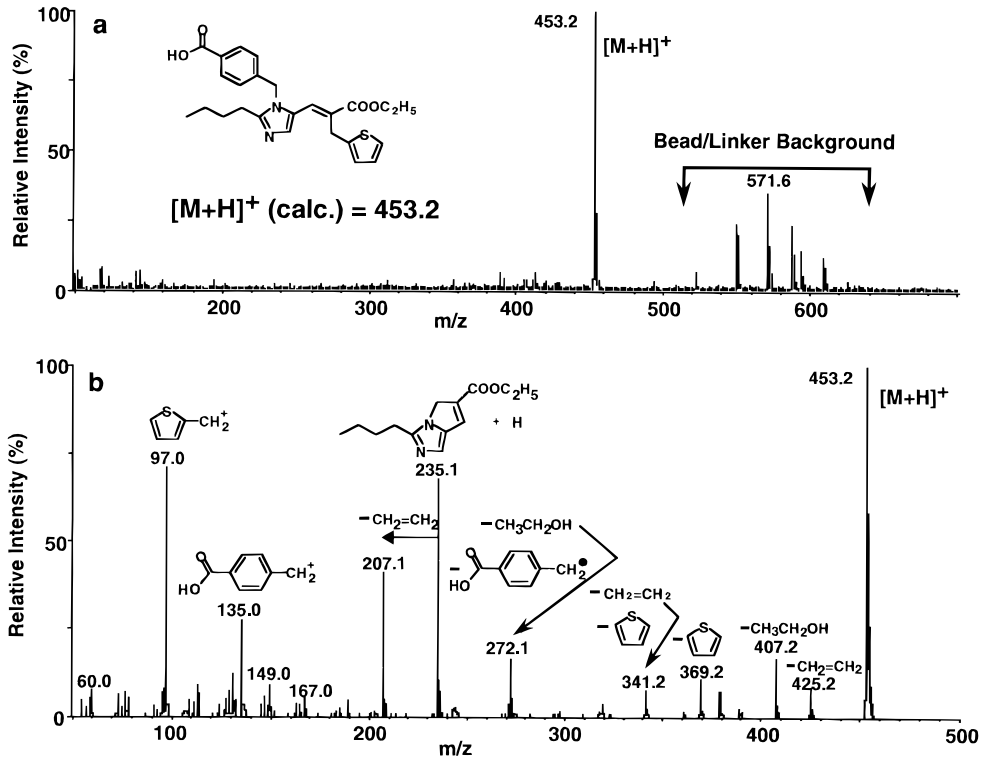

Figure 1. Electrospray mass spectrum (a) and electrospray MS/MS spectrum (b) of angiotensin II antagonist. Approximately 10% of the total analyte released from a single Sasrin bead by 1% TFA in methylene chloride was used for each analysis. The MS/MS spectrum was derived by collisional activation in a Sciex triple−quadrupole mass spectrometer of *m*/*z* 453.2, the $(M + H)^+$ of the angiotensin II antagonist.

For MALDI-MS experiments, a single bead was placed on a stainless steel substrate. The target molecule was clipped from the bead by exposure to 3 μL of a 1% TFA solution in CH_2Cl_2 and activated for MALDI by addition of 0.5 μL of an acetone solution of 2,5-dihydroxybenzoic acid (DHB). Spectra were recorded using a Fisons VG TofSpec-SE reflectron analyzer after desorption using a 337-nm pulsed N_2 laser and extraction at 25 kV. Spectra from ~40 laser pulses were summed, and the centroids of the unsmoothed data determined at 50% peak height.

Structural information may be discerned from MALDI-MS by analysis of metastable ions formed in the field-free drift region of the time-of-flight analyzer.[16−19] To observe fragment ions, a Bradbury−Nielsen ion gate[20] set to pass ~15 mass units was centered about the parent ion at *m*/*z* 453.2. The metastable ion mass spectra were then constructed by combining seven consecutive overlapping mass scale segments (30 laser pulses/segment), each representing ~25% energy change from the previous segment. Segments of the metastable ion spectra were combined, the data smoothed and the centers of mass determined at 75% peak height and then externally mass calibrated vs substance P, amino acids 2−11 (δ) by the data system.

The TOF-SIMS measurements were performed by placing a single angiotensin-coated bead directly onto a silicon wafer. The wafer was placed in a chamber saturated with TFA and CH_2Cl_2 vapor in equilibrium with 15% TFA in CH_2Cl_2 for ~2 h. This procedure cleaves the chemical bond between the angiotensin molecule and the Sasrin linker without physically removing it from the bead and allows some of the molecules to be sputtered from the surface intact.[9] After treatment, the wafer was directly transferred to a Kratos Prism TOF-SIMS instrument (Manchester, UK) for analysis. Secondary ions were produced using a 25-keV Ga^+ ion pulse of 7-ns duration, focused to a spot size of ~150 nm. The ions were extracted at 7.2 keV into a reflectron TOF analyzer. Ion beam dose is a critical issue for TOF-SIMS measurements in order to avoid chemical damage to the sample surface.[21] For the angiotensin samples examined here, the dose was kept below 8 $\times 10^{11}$ Ga^+ ions/cm^2. Electrical charging of the insulating bead was compensated by flooding the surface with 30-eV electrons for 50 μs after each Ga^+ ion pulse. Direct imaging of a single bead was achieved by rastering the primary beam across the target and monitoring the intensity of the $(M + H)^+$ signal as a function of beam position.

RESULTS AND DISCUSSION

Each of the MS methods presented here yields information about the structure and composition of a target molecule that is associated with a single polystyrene bead. This feature is illustrated for the AII receptor antagonist synthesized on bead and examined by each method.

The results for ESMS are shown in Figure 1. The extract from the bead yields a strong $[M + H]^+$ ion signal at *m*/*z* 453.191, near the theoretical value of *m*/*z* 453.185. Other unidentified peaks associated with the bead or the Sasrin linker are also seen in the *m*/*z* 500−600 range. Structural identification is possible using the MS/MS feature of the triple−quadrupole detector as

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Table 1. Calculated and Observed Masses of Fragment Ions Observed for Angiotensin II

composition	calcd mass, Da	mean measd mass ± SD (error[a]), Da		
		ESMS[b]	MALDI[c]	TOF-SIMS[d]
C_5H_5S	97.0112	96.97 ± 0.02 (+0.04)	97.04 ± 0.01 (−0.03)	97.015 ± 0.006 (−0.004)
$C_8H_7O_2$	135.0446	135.04 ± 0.02 (+0.01)	135.09 ± 0.01 (−0.05)	135.045 ± 0.007 (0.000)
$C_{11}H_{15}N_2O_2$	207.1133	207.11 ± 0.01 (0.00)	207.03 ± 0.01 (+0.08)	n.o.[e]
$C_{13}H_{19}N_2O_2$	235.1447	235.14 ± 0.01 (+0.01)	235.14 ± 0.00 (0.00)	n.o.
$C_{15}H_{16}N_2O_1S$	272.0983	272.12 ± 0.01 (−0.02)	272.07 ± 0.02 (+0.03)	n.o.
$C_{17}H_{19}N_2O_2$	283.1446	n.o.	283.09 ± 0.03 (+0.06)	283.144 ± 0.015 (+0.001)
$C_{19}H_{21}N_2O_4$	341.1501	341.15 ± 0.01 (0.00)	341.11 ± 0.05 (+0.04)	n.o.
$C_{21}H_{25}N_2O_4$	369.1814	369.17 ± 0.01 (+0.01)	369.16 ± 0.03 (+0.03)	n.o.
$C_{23}H_{23}N_2O_3S$	407.1429	407.15 ± 0.01 (0.00)	407.12 ± 0.02 (+0.02)	n.o.
$C_{23}H_{25}N_2O_4S$	425.1535	425.19 ± 0.01 (−0.03)	425.10 ± 0.04 (+0.06)	n.o.
$C_{25}H_{29}N_2O_4S$	453.1848	453.191 ± 0.004 (−0.007)	453.18 ± 0.01 (+0.01)	453.183 ± 0.008 (+0.002)

[a] Error = calculated mass − mean measured mass. [b] Fragment masses were determined in six independent MS/MS experiments; the molecular ion mass was determined in three independent MS experiments [c] Fragment masses were determined by analysis of metastable ions in three independent experiments; the molecular ion mass was determined in three independent reflectron MS experiments. [d] Values were determined by the average of three independent measurements. [e] n.o., not observed.

shown in Figure 1b. There are a large number of fragment ions which are characteristic of the molecular structure of the parent. The important assignments are denoted in the figure and are summarized in Table 1. Both spectra were recorded with a total of 20% of the extracted molecules. Moreover, we note that with the recent addition of microsprayers that operate efficiently at flow rates as low as 50 nL/min (vs the 2 μL/min used here) 2 orders of magnitude higher sensitivity is feasible, effectively eliminating any practical restrictions on the number of analyses that may be performed on a single bead.[22]

Similar results are obtained using MALDI-MS in combination with analysis of metastable ions for structural assignments. The mass spectrum produced by averaging the results from 41 laser shots directed toward an identical area of the bead is shown in Figure 2a; essentially identical spectra were obtained regardless of where the laser was aimed on the target. The $[M + H]^+$ ion peak is found at m/z 453.18 and is 0.4 mass unit wide (full width at half-height), indicating that the mass resolution is nearly 1300. A resolution of >4000 is usually achievable on this and other high-performance reflecting time-of-flight mass spectrometers.[23,24] The lower resolution obtained here is due to the use of DHB as a matrix. This matrix requires the use of somewhat higher laser energies than are required for other matrices such as α-cyano-4-hydroxycinnamic acid which reduces the observed resolution. However, DHB is well suited for the analysis of small molecules because it produces less background at low m/z than other matrices.[25] The larger peaks below m/z 200 are attributable to DHB. The metastable ion spectrum of the AII antagonist is shown in Figure 2b. The spectrum bears a striking resemblance to the ESMS/MS spectrum and can be readily utilized to determine the structure of the original molecule. The major fragment ions are enumerated in Table 1.

While ESMS and MALDI-MS are similar in their experimental protocols and in their results, the TOF-SIMS spectrum provides somewhat different but chemically specific information. The spectrum of AII antagonist obtained directly from a single bead previously exposed to TFA vapor is shown in Figure 3. The quasimolecular ion is observed at m/z 453.183. Fragment ions are present without the application of MS/MS or metastable analysis techniques, some of which are distinct from those observed via ES or MALDI-MS. These fragments are not generally the same as those found with other MS techniques because of the different mechanism associated with the sputtering event.[26] The major fragments along with their structural assignments are reported in Table 1, where they can be directly compared with those found from ES and MALDI measurements.

As a consequence of the small diameter of the Ga^+ ion beam, it is possible to spatially resolve the composition of a single bead in the presence of other beads coated with other library members. This chemical mapping has been recently demonstrated using peptide libraries.[9] As shown in Figure 4, similar images are possible for our model antagonist system. Spatial resolution may be an important attribute if screening procedures require a parallel assay of large numbers of active beads.

Another powerful feature of MS for directly addressing active beads alluded to above involves high mass accuracy measurements. For example, in a 1000-component library synthesized in three steps from three mixtures of 10 different functionalities, mass accuracy of ±0.5 Da yields ~11 candidate structures with indistinguishable molecular weights. A mass accuracy of ±0.05 Da reduces this redundancy to nine and an accuracy of ±0.01 Da reduces it still further to only four possible structures. As shown in Table 1, parent ion mass accuracies of ±0.01 Da or better are achieved (at comparable levels of precision) by each of the MS methods, allowing any one of them to be utilized in this approach to eliminate candidate structures. The fragmentation patterns may then be used to uniquely resolve the remaining ambiguities.

All of the MS methods appear to have adequate sensitivity for addressing single beads. There are several interesting features, however, that distinguish each approach. Both ES and MALDI-MS yield intense quasimolecular ion peaks which lead to direct, unambiguous molecular weight determinations using a small percentage of the molecule associated with a single bead.

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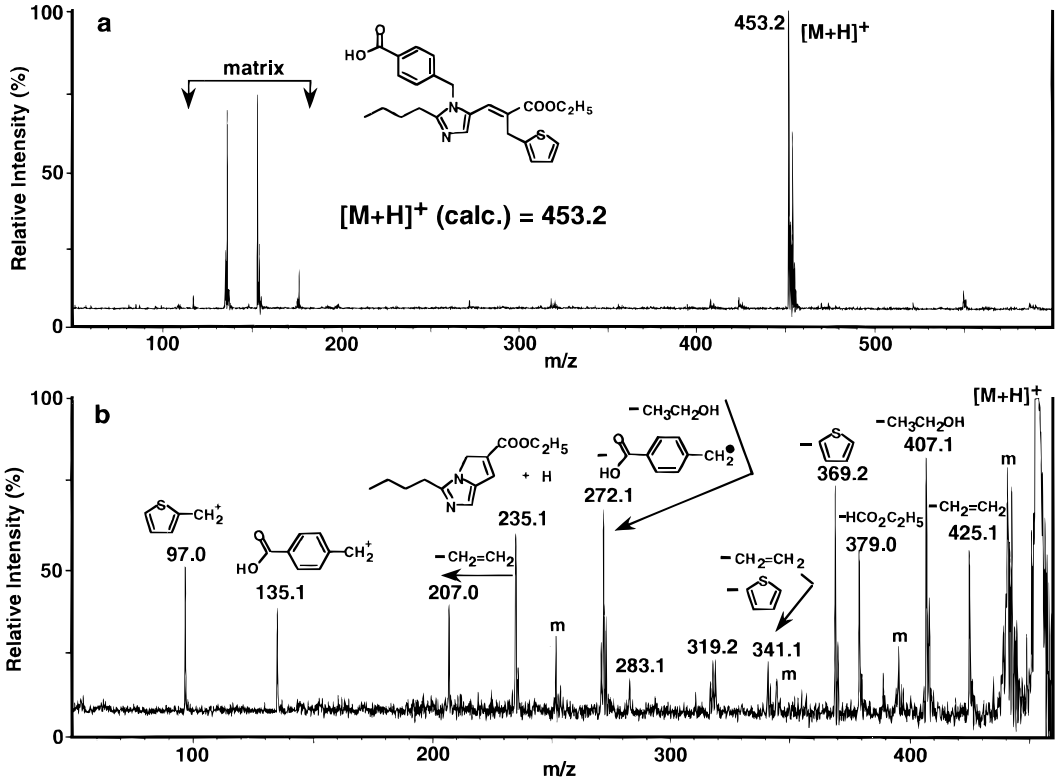

Figure 2. MALDI mass spectrum (a) and metastable ion MALDI mass spectrum (b) of angotensin II antagonist. For each analysis, a single Sasrin bead was transferred to the MALDI target and analyte was released using 3 × 1.0 μL 1% TFA in methylene chloride. The solution was allowed to air-dry prior to adding a 1-μL aliquot of cleavage reagent. 0.5 μL of matrix solution (5 mg of DHB/mL of acetone) was deposited over the bead onto the target and MALDI analysis performed using photon irradiation from a 337-nm pulsed nitrogen laser and an accelerating voltage of 25 kV. The normal MALDI spectrum (a) of the angiotensin II antagonist in DHB matrix shows a significant level of what appears to be reduction of the molecule. The metastable ion spectrum (b) was acquired using α-cyano-4-hydroxycinnamic acid (9 mg/mL 10:10:1 EtOH/CH_3CN/H_2O) in order to generate better fragment ion intensity. Peaks labeled "m" in the spectrum are matrix-derived whose precursors fall within the ±7.5-Da Bradbury−Nielson gating window as determined by acquiring a matrix background spectrum under identical conditions.

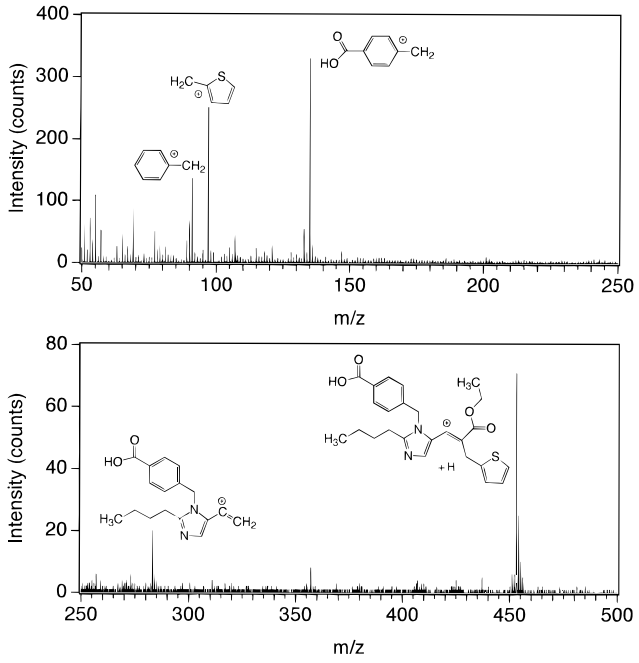

Figure 3. TOF-SIMS mass spectrum of angiotensin II antagonist on a Sasrin bead after cleavage by exposure to TFA vapor for 2 h.

Structural determinations are possible from studies of the fragment ions generated in MS/MS or metastable ion analyses. ESMS is well suited for automation and sequential analysis of many target beads. For example, single beads can be placed in autosampler vials and cleavage agent added either manually or robotically. Alternatively, beads that have been bioassayed using 96-well microtiter filter plates can easily be cleaved and eluted a final time with the filtrate robotically transferred to autosampler vials for MS analysis. Several commercial ESMS systems are readily adapted to high-throughput analysis using an autosampler, with multiple analyses per vial. Some systems can even be programmed for multiple analyses from a single injection (e.g., full scan and MS/MS). MALDI-TOFMS sample preparation might also be automated robotically with the microtiter plate filtrate transferred robotically to the MALDI sample target, with matrix added at an appropriate point. Sample target positioning is already under data system control, but automation of the actual MALDI analysis may prove to be more difficult due to the need to find areas for a given sample preparation that will produce ions and to adjust the laser energy to yield high-quality data. Both problems may be ameliorated by the use of uniform thin matrix films,[24] but this will require further investigation.

TOF-SIMS is less widely used for this type of analysis; consequently, the measurements are at a more experimental stage; nevertheless it is clear that the technique presents a number of interesting assaying possibilities. The molecular ion is not usually the most intense ion in the spectrum, although it is generally associated with the peak that occurs at the highest mass. The desorbed ions are generally thought to arise from the first few layers of the solid. For solid phase synthesis on beads, it is well-

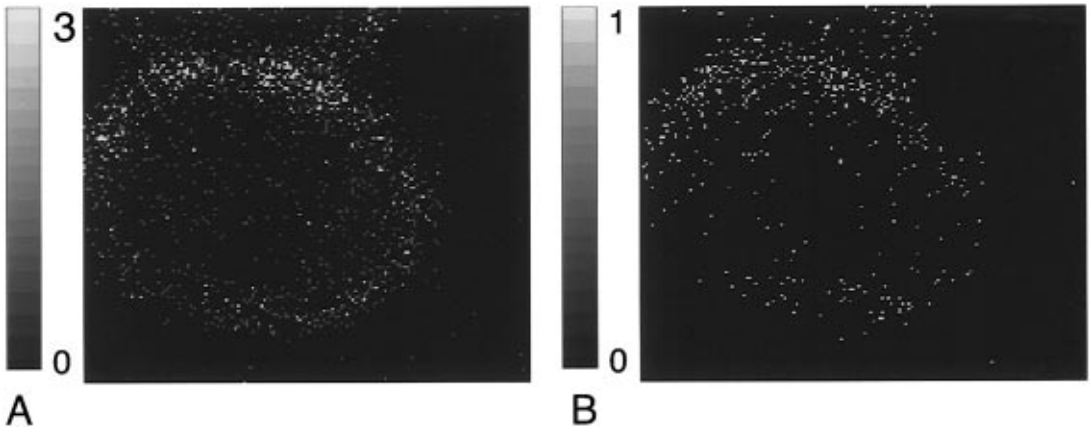

Figure 4. TOF-SIMS images of the $(M + H)^+$ ion (*m*/*z* 453.2) (A) and the *m*/*z* 135 fragment ion (B) from angiotensin II antagonist on a Sasrin bead after cleavage by TFA vapor. The bead is spherical, but appears as an ellipse because the image is the result of projecting a three-dimensional object (a sphere) on to a plane from a 45° angle. The expected elongation along the axis of the incident ion gun is a factor of 1.41 with respect to the other axis; the same elongation is observed. The second feature worth mentioning is the apparent variation in the distribution of the sample across the surface of the bead even though the bead is uniformly coated. The apparent nonuniformity is produced by two factors, the change of the angle of incidence of the primary ion beam as it sweeps across the bead surface, which affects the amount of secondary ions desorbed, and the different extraction voltage at different heights of the bead (a 60-μm bead has a 150-V drop from bottom to top) which affects which ions are focused onto the detector. The combination of the two factors produces a variation of the signal across the bead.

known that the bulk of the material resides inside the sphere.[27] For example, a bead of 50 μm in diameter yields ~100 pmol of product. The surface of a smooth bead, however, accommodates only 10 fmol of material (for a 10 Å × 10 Å molecule). Hence, it may be possible to perform many partial extractions from a particular bead and still retain adequate sensitivity for analysis. Another feature involves high mass accuracy measurements. For TOF-SIMS, mass accuracy of 1−10 ppm is expected.[21] For our model system, the measured mass is 453.183 ± 0.008 Da (±20 ppm), which differs from the expected mass by 0.002 Da (4 ppm). This precision and accuracy allows mass redundancies to be reduced or eliminated as a result of a single mass measurement. This feature is particularly important for non-peptide libraries where complicated three-dimensional structures may be difficult to determine from fragmentation patterns alone. The TOF-SIMS spectrum also provided a number of significant fragments where mass was accurately determined. Thus, while the number of fragments generated was fewer than in ESMS/MS and from the MALDI metastables, the chemical structure of the target molecule could be defined. It should be remembered that with appropriate instrumentation metastable ions can also be studied in TOF-SIMS.

Finally, the imaging capability of TOF-SIMS allows for the possibility of examining a large number of beads arrayed onto a single indexing grid. Moreover, other possible approaches to constructing libraries are under development. For example, Si chip technology is being used to build library arrays of molecular variants.[28] Imaging TOF-SIMS would be well-suited to confirming the structures of compounds in these arrays that have been shown to have biological activity.

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CONCLUSIONS

Our analysis would suggest that any one of the MS techniques could fulfill the requirements for direct bead analysis of combinatorial libraries, but that several interesting features distinguish each approach. MALDI-MS, TOF-SIMS, and ESMS each yield measurements of the parent ion with sufficient precision and mass accuracy (<±0.01 Da) to eliminate candidates with similar molecular weights. While TOF-SIMS provided more accurate fragment ion masses, MALDI-MS and ESMS provided a greater number of structurally informative fragments. All of the approaches have the sensitivity to interrogate a single bead. The TOF-SIMS because of its spatial resolution can resolve the composition of a single bead from other library members in a mixture of beads providing a procedure for a parallel assay of a bead matrix. ESMS with flow injection analysis is well suited for automation and sequential analysis of many target beads. Automated analysis by MALDI-MS or TOF-SIMS may also be adapted to robotic operation, although this has not yet been practically demonstrated. All of the MS techniques described would appear to largely eliminate the need for tagging methods to identify the chemical composition of a ligand synthesized on a solid support by combinatorial methods. Finally, we note that further improvements in exact mass assignments for analysis of ligands synthesized on beads have recently been demonstrated through the use of Fourier transform ion cyclotron resonance MS.[29] The capability of FTICR and its amenability to a number of ionization methods warrants continued investigation.

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