Peptide Sequencing Using a Patchwork Approach and Surface-Induced Dissociation in Sector-TOF and Dual Quadrupole Mass Spectrometers

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Surface-induced ion activation in combination with a database search strategy based on the Patchwork concept is applied to the determination of peptide sequences. Surface-induced dissociation (SID) is performed in a tandem quadrupole mass spectrometer and in a hybrid sector/time-of-flight mass spectrometer in order to evaluate the importance of accurate mass analysis of the SID fragment ions for peptide identification. The modified Patchwork approach is based on piecing together the peptide blocks in a bidirectional way, simultaneously using low-mass fragments originating from the C-terminus and N-terminus of the molecule, and relying on the measurement of the peptide's molecular weight with moderate mass accuracy. The results from this analysis are used as search filters in MASCOT's (http://www.matrixscience.com) Sequence Query search engine, with the simultaneous addition of the full MS/MS peak list. SID is performed with collision targets coated with pure and mixed composition self-assembled monolayers produced by fluorocarbon and hydrocarbon alkanethiolate solutions of varying chemical composition. The resulting MS/MS spectra produced on pure and mixed hydrocarbon SAMs are submitted to the modified version of Patchwork sequencing. It is found that hydrocarbon surfaces improve the relative abundance of larger fragments. Under the moderate mass accuracy conditions (±0.3 u) offered by our linear-TOF-SID instrument, it is found that increasing the abundance of larger fragments dramatically improves the sequencing scores. (J Am Soc Mass Spectrom 2003, 14, 1387–1401) © 2003 American Society for Mass Spectrometry

Tandem mass spectrometry (MS/MS) coupled with liquid chromatography has become a routine analytical method for the identification of proteins through peptide sequencing in combination with automated database searches [1, 2]. By far, the most common ion activation technique currently employed to induce fragmentation of peptide ions is low energy collision induced dissociation (CID) in which mass-selected ions are collided with neutral gas atoms (Ar for example). However, there is still much improvement needed in the coverage obtained for the protein sequences identified by CID MS/MS. As an example, recent work on a tryptic digest of a cellular membrane protein fraction showed that a significant fraction (~24%) of the peptide MS/MS spectra generated by CID in a quadrupole-ion trap remained unmatched to the correct sequence [3]. This failure in identifying the correct peptide sequence is a common problem, rather than an exception, resulting from a variety of issues in the MS/MS spectra (i.e., information too complex for automated sequencing, low signal intensity, insufficient fragmentation etc.). The challenging task of matching unknown peptides to their correct structure has motivated scientific and technological advances in many areas related to proteomics including separations science [4–6], mass spectrometry instrumentation [7], automated sequencing algorithms [8], statistical analysis of peptide fragmentation trends in MS/MS spectra [9], and gas-phase peptide fragmentation mechanisms [10].

Although automated peptide sequencing using collision induced dissociation (CID) is currently in widespread use for protein identification efforts, alternative activation methods such as surface induced dissociation (SID) should be explored to determine whether they provide similar or complementary fragmentation information. One of the potential advantages of SID is that, at some collision energies, it deposits higher

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average internal energies to mass-selected peptide ions upon activation when compared to low-energy CID [11, 12]. This lower efficiency can be attributed to the fact that multiple-collision low-energy CID suffers from collisional deactivation of the internally excited ion as a competing process [12]. Ions formed from large biomolecules require larger amounts of internal energy than small organic ions to achieve fragmentation because they contain more internal vibrational and rotational modes over which the internal energy may be distributed. Tandem mass spectrometry dissociation of such large biomolecules can provide structural information, but CID of singly charged ions has an upper limit of about 3 kDa [13]. With the multiply charging provided by electrospray, this limit appears to be removed, and CID dissociation for very large ions (150 kDa) has been reported [14]. It has been previously shown that SID can also efficiently fragment very large biomolecules such as doubly and triply charged insulin B chain (M_w = 3468.7 g mol^{-1}) [15]. Due to its increased kinetic-to-internal energy transfer efficiency, SID might be a promising tool for ion activation either by itself or by preactivating the precursor ions by CID. Although SID in combination with CID is a strategy that still has to be investigated, it could be appealing in the case of large, singly charged ions or extremely large noncovalent complexes that do not fragment as much as desired by CID. Another advantage of SID is that the relationship between the energy deposited into the precursor ion and the laboratory collision energy is linear over a larger range of energies than for low-energy CID [12]. It has also been shown that SID produces ions with a narrower spread of internal energies than high energy CID [16] and with a similar spread to multiple-collision low-energy CID [11]. In order to discover the full potential of this technique, further research towards practical uses of SID needs to be performed.

In order to use SID spectra for protein identification in "bottom-up" proteomics [1–8], automated peptide sequencing from raw SID MS/MS data must be first realized. The ultimate goal of the research presented in this paper is to achieve peptide sequencing with SID-generated MS/MS spectra combined with database-searching algorithms widely available to the public. Such algorithms include Protein Prospector's MS-Tag Unknome [17] and Mascot's Sequence Query [18] which we have used in conjunction with a modified version of the previously reported Patchwork peptide sequencing approach [19]. It is well known that accurate mass analysis is a factor in peptide sequencing applications. The effect of mass accuracy on a modified Patchwork sequencing approach is investigated here in combination with the effect that the chemical composition of the target surface has on the quality of the obtained spectra.

Materials and Methods

Projectile Ions and Target Surfaces

Doubly deionized water (DIW) was obtained from a MilliQ water purifying system (Millipore, Bedford, MA). YGGFL (MW_{MH} = 556.28 u), VPDPR (MW_{MH} = 583.32 u), DSDPR (MW_{MH} = 589.26 u), YIGSR (MW_{MH} = 595.32 u), SIGSLAK (MW_{MH} = 675.40 u), DLWQK (MW_{MH} = 689.36 u), ASHLGLAR (MW_{MH} = 824.47 u), PPGFSPFR (MW_{MH} = 904.47 u), CDPGYIGSR (MW_{MH} = 968.08 u), VQGEESNDK (MW_{MH} = 1006.02 u), FSWGAEGQR (MW_{MH} = 1038.11 u), RPPGFSPFR (MW_{MH} = 1061.23 u), (Sigma, St Louis, MO) and YGGFLR (MW_{MH} = 712.38 u), YGGFLRR (MW_{MH} = 868.48 u) (American Peptide, Sunnyvale, CA) were used as received from the vendor. All other reagents and solvents were purchased from Sigma. LMYPTYLK (MW_{MH} = 1029.29 u) was synthesized in our laboratories.

The matrix mixture used for FAB ionization in the sector-TOF consisted of a 50:25:25:1 mixture of glycerol, thioglycerol, 3-nitrobenzyl alcohol, and trifluoroacetic acid. In the dual quadrupole-SID (QQ) instrument, peptide ions were produced by electrospray ionization from a 70:30 methanol:DIW solution with 1% acetic acid added.

Single composition self-assembled monolayers (SAMs) were prepared by the spontaneous assembly of 2-(perfluoro-decyl)ethanethiol (C12F10) and dodecanethiol (C12) chemisorbed on gold-coated glass slides. Gold surfaces (100 nm of vapor-deposited gold on glass with a 50 Å Ti adhesion layer) were obtained from Evaporated Metal Films (Ithaca, NY). Slides were cleaned for 25 min using a UV-cleaner (Boekel, Feasterville, PA) and rinsed three times with ethanol. Mixed SAMs were prepared by mixing the appropriate volumes of 1 mM solutions of the pure thiols in ethanol. Mixed C12F10:C12 surfaces were prepared by immersing a clean Au surface in ethanol solutions containing the corresponding thiols in 100:0, 70:30, 50:50, 30:70, and 0:100 molar ratios. The reaction time was 24 h in all cases, at room temperature. After the reaction took place, the slide was rinsed with ethanol three times and sonicated (model 1200 Branson, Danbury, CT), for 10 min in clean ethanol. The slides were dried with dry nitrogen before they were mounted on the surface holder and inserted into the vacuum chamber of the MS. For more details on surface preparation and SAM synthesis see reference [20].

Sector-TOF Mass Spectrometer

Figure 1a shows a diagram of the in-line sector-TOF instrument. The optical elements used for CID ion activation (not used in the present work) have been omitted for simplicity and are described in detail in our earlier paper describing the development of the instrument [21]. Timing of the pulses to control the TOF

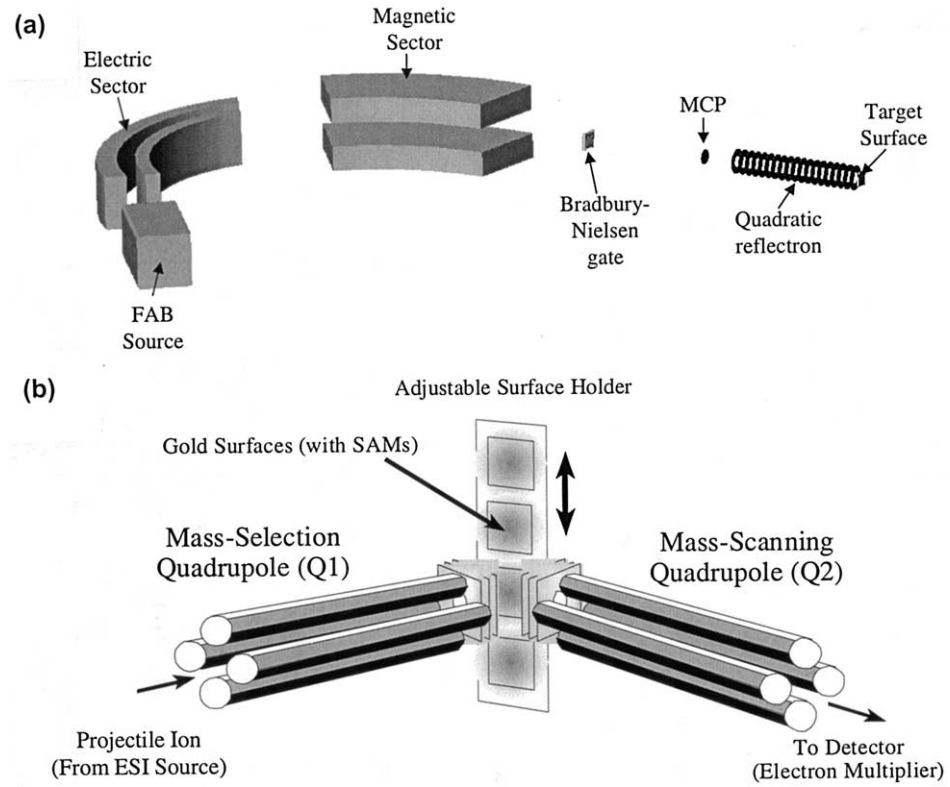

Figure 1. (**a**) Sector-TOF SID instrument and (**b**) dual quadrupole SID instrument used to generate low-energy ion-surface collisions.

analyzer was performed with a digital delay generator (DG535, Stanford Research Systems, Sunnyvale, CA). Spellman high voltage electronics (Plainview, NY) were used to produce the static DC potentials and Behlke fast high voltage switches (Eurotek, Morganville, NY) were used to drive the Bradbury-Nielson type ion gate [22] and the surface extraction pulse. The precursor ions entering the TOF analyzer were mass and energy selected in a doubly-focusing JEOL HX110A sector (EB) mass spectrometer (Peabody, MA). FAB with a conventional Xe gun was used as the ionization method producing only singly charged ions. Base pressure of the TOF chamber during SID analysis was approximately 5×10^{-8} torr. The entrance and exit slits of the sector analyzer were set so as to achieve unit mass selection for precursor ions of *m/z* up to 700 u allowing for the use of monoisotopic masses for calibration purposes in this mass range. For larger peptides, unit mass resolution cannot be achieved together with slit settings that provide a good signal-to-noise ratio (SNR) at the TOF stage and thus, average masses were used when calibrating of the TOF spectra. A PC running Microsoft Windows 95, equipped with a 1 GHz TDC board (model 9805, Precision Instruments, Knoxville, TN) was used for data acquisition. Acquisition times ranged between 64 s and 512 s that translate to an average of 1,152,000 sweeps per spectrum. The MCP coaxial detector was operated at 1900 V; a higher voltage setting increased sensitivity with a consequent loss in resolution due to detector ringing. Larger peptides (>700 u) required longer acquisition times to compensate for the decreased intensity of the ion beam produced by the FAB source. Spectra were calibrated using in-house software developed by JEOL.

Mass calibration was performed by running a known compound (usually singly charged YGGFL) and using a standard, two-point calibration procedure locking on a low mass fragment (e.g., im-L) and on the surviving precursor ion. The mass-to-charge axis was recalibrated for new samples using a known fragment as the lock mass. The recalibration procedure was repeated iteratively until the best match with the expected *m/z* values was obtained. Theoretical *m/z* values were taken from the MS-Product module included within the Protein Prospector software package (http://prospector.ucsf.edu). SID spectra were smoothed using a 10-point-window adjacent averaging routine provided by Origin (version 6.0). Baseline subtraction, if needed, was also performed in Origin.

Surface-Induced Dissociation in the Sector-TOF Instrument

Surface-induced dissociation (SID) has been performed in a variety of instrument types, including QQ, sector-TOF, triple quadrupoles, FT-ICR and TOF/TOF spectrometers [23]. Although complex

from the instrumental point of view, the hybrid sector-TOF instrument has the advantage of eliminating any uncertainties in the primary ion beam kinetic energy due to the efficient mass and energy selection offered by its dual focusing geometry [24]. In the particular case of peptide sequencing applications, the TOF analyzer has the advantage of offering a high quality fragment mass spectrum, due to its inherent mass accuracy [25]. Many research groups have shown that SID can be performed at the back end of the reflectron of TOF analyzers providing an inexpensive setup that can be implemented in existing commercial instruments [21, 26–30]. It is worth mentioning that this setup, although simple, suffers from lower mass resolution than reflectron TOF analyzers.

The operation of the sector-TOF instrument can be briefly described as follows: Ions produced by a FAB source are accelerated to 5 keV and subsequently mass and energy selected. During MS/MS operation, the sector electron multiplier is turned off and the ion beam is allowed to pass through the flange that connects the sector part of the spectrometer to the TOF analyzer. Upon entering the TOF chamber, the ion beam is cylindrically shaped by a set of quadrupolar lenses and 800 ns-long ion packets are produced by an "interleaved comb" bipolar ion gate at a frequency of 4 kHz. The voltage applied to the ion gate wires was 250 V in all cases. After the ion packet is formed, it travels into an offset parabolic field reflectron, whose voltage is set approximately 150 V lower than the DC bias voltage applied to the SID surface. During the SID mode of operation, the reflectron does not turn around the ion packet and only acts as a retarding and focusing stage before the collision. This setting allows the energy and mass-selected ion packet to pass through the "reflectron" region and strike the SID collision target. Conversely, after the collision takes place, the "reflectron" functions as an offset acceleration stage. The SID collision energy can be controlled by adjustment of the potential difference between the ion source (nominal 5 kV acceleration potential) and the surface DC bias voltage. The zero collision energy can be accurately determined by finely measuring the turning point of the ion packet, i.e., the potential at which the precursor ion time-of-flight is slightly shifted to shorter values, without hitting the surface. In general, collision energies between 10 and 90 eV were assayed. The spectra acquired using 45 eV collisions were used for comparison with the QQ-SID spectra. After ions collide with the surface, a 200 ns delay is introduced followed by a 500 V voltage pulse added to the surface DC bias, thus ejecting the ions back into the "reflectron" (500 V cm^{-1} between extraction grid and surface electrode). The timing of this surface pulse is critical and is synchronized with the opening of the ion gate. The duration of this pulse can be roughly estimated as equal to the flight time between the gate and the surface plus the extraction delay time. This sort of "delayed extraction" produced mass accuracies between ± 0.1 and ± 0.3 u (250 ppm on average) for both precursor and fragment ions, as is shown in Figure 2. The surviving precursor ions and the newly formed fragment ions are detected by a coaxial MCP detector.

Tandem Quadrupole Mass Spectrometer

The tandem quadrupole (QQ) instrument used throughout this work is specifically designed for low-energy ion-surface collisions (Figure 1b). The instrument consists of two 4000 u quadrupoles (ABB Extrel, Pittsburg, PA) that are arranged in a 90° geometry with the surface positioned at the intersection of the optical paths, producing a collision angle of 45° with respect to the surface normal. The surface holder can be rotated to optimize ion collection efficiency. In general, angles between 45° and 50° degrees gave SID spectra with the maximum SNR. The surface holder can accommodate up to four different SAM-coated slides, allowing one to perform SID on a variety of chemical substrates during the same run. The surface can be easily switched by moving the surface holder perpendicularly to the plane that contains both quadrupoles. A typical SID experiment for a singly charged ion is carried out in different steps. First, the precursor ion is mass-selected with the first quadrupole. Then the ion beam is forced to collide against the surface by a source-surface potential difference equivalent to the laboratory collision energy. Following the collision, the resulting fragments and remaining precursor ion are collected and focused by a set of three independent planar lenses into the second quadrupole, which performs a final mass scan. Ionization was performed by electrospray, using a simple heated capillary-skimmer cone source. The electrospray needle was held at 4.2 kV, the inlet metal capillary voltage was typically set to 110 V and the skimmer voltage was set to 90 V. Samples were infused at 3 μL min^{-1} using a SAGE model 361 syringe pump (Thermo Orion, Beverly, MA). The collision energy was determined by the potential difference between the skimmer cone (fixed) and the surface DC bias (variable). The instrument base pressure during ESI operation was $2x10^{-5}$ torr. Only singly charged precursor ions were fragmented by SID in the QQ instrument, due to the fact that the sector-TOF FAB source produces only singly charged ions, which does not enable a direct comparison between SID experiments for multiply charged ions using both instruments.

Results and Discussion

Effect of Mass Accuracy on the Peptide Sequencing by SID with MS-Tag Unknome

MS-Tag Unknome (local copy) is an algorithm included in the Protein Prospector software package for the automated sequencing of peptides from MS/MS data. The basic principles of operation for MS-Tag Unknome

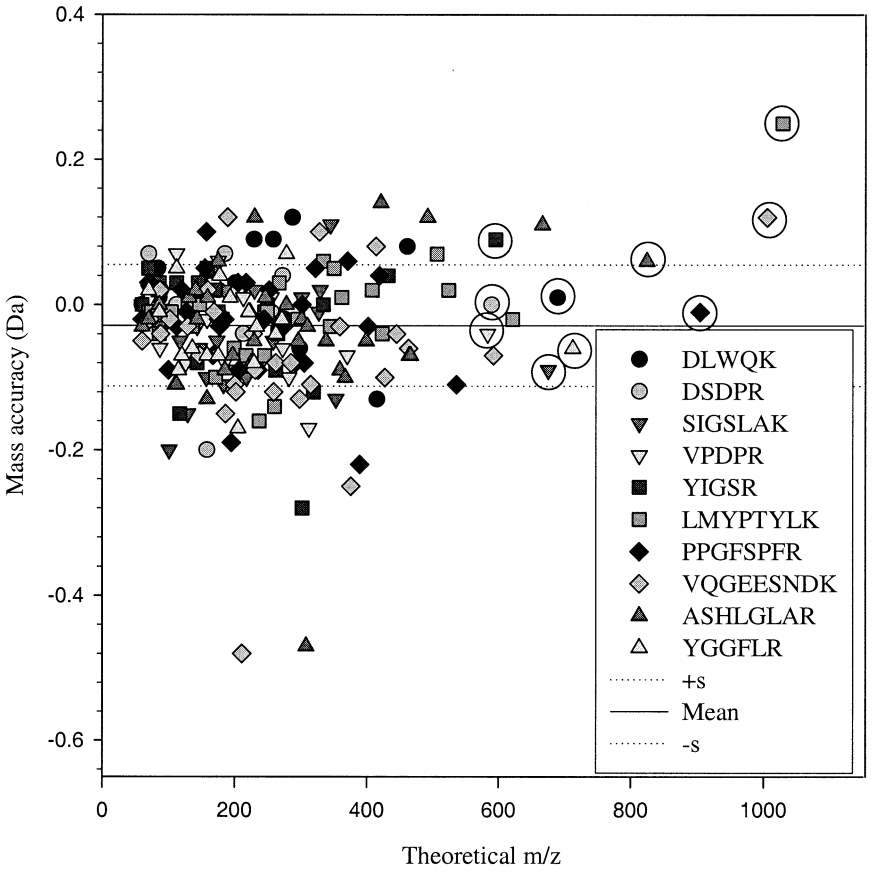

Figure 2. Mass accuracy for parent (encircled) and fragment ions as a function of their mass observed in the sector-TOF SID MS/MS spectra.

include calculation of all theoretical elemental compositions for the unknown peptide sequence on the basis of a moderately accurate precursor ion *m/z* ratio, followed by the generation of appropriate amino acid residue combinations using the calculated elemental compositions and compositional information indicated in the MS/MS spectra by the presence or absence of immonium ions. Because SID MS/MS spectra have a wealth of immonium ion information present, this approach is an attractive candidate for automated peptide sequencing. For each candidate peptide sequence generated by MS-Tag Unknome, the algorithm calculates theoretical fragment ion *m/z* values for user defined fragment ion types. These theoretical fragment ion *m/z* values are then compared with fragment ion *m/z* values present in the experimentally measured MS/MS spectrum and the candidate sequences are ranked in the order of increasing number of unmatched experimental fragment ion *m/z* values. Because the implemented scoring routine (ranking candidates based on the number of unmatched fragments) is overly simplistic, multiple candidate sequences are frequently given the same ranking. For the present work, we have defined a custom quality quotient, *Q*, which provides both a quality measure for the degree of matching of the MS/MS spectrum and also for the number of false positive results:

$$Q = \frac{([\text{Number of ions matched/number of total ions}] \times 100) \text{ for the correct sequence}}{(\text{Total number of unique peptide sequences ranked higher than or equal to correct sequence.})}$$

As previously described, immonium ions present (or absent) in the MS/MS spectrum were used to determine the presence (or absence) of amino acid residues in the unknown peptide sequence. MS-Tag Unknome automatically assigns the presence of a residue in the sequence by detecting the presence of the corresponding immonium ion *m/z* value in the list of experimental fragment ion *m/z* values submitted to the algorithm. The absence of a residue is a selection criterion manually applied by careful inspection of the MS/MS data. Although, in theory, immonium ions can be formed for every residue, there are certain residues that consistently produce strong immonium ion peaks in SID MS/MS spectra. These residues include proline (70 u), valine (72 u), threonine (74 u), isoleucine/leucine (86 u), aspartic acid (88 u), lysine/glutamine (84 u and 101 u), methionine (104 u), histidine (110 u), arginine (70, 112 u), phenylalanine (120 u), and tyrosine (136 u). In

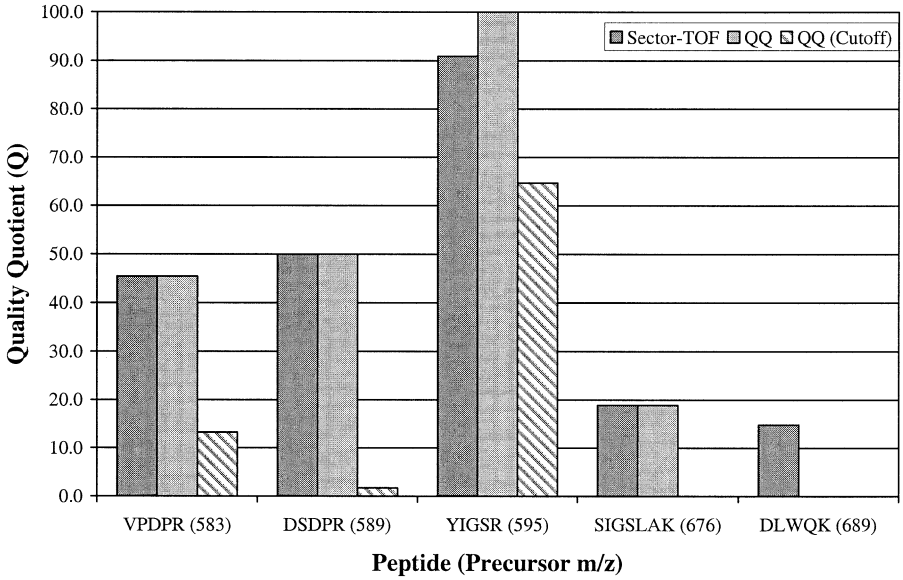

Figure 3. MS-Tag Unknome results for automated interpretation of spectra for small peptides (collided with C12F10 target surface) acquired with sector-TOF-SID (+/− 0.3 u; dark solid bars), dual quadrupole-SID (+/− 1.0 u; light solid bars), and dual quadrupole-SID with fragment ions excluded that have *m/z* values higher than largest sector-TOF-SID fragment ion (+/− 1.0 u; striped bars).

our experience, the occurrence of these immonium ions in SID spectra is consistent with the presence of the corresponding amino acids and consequently, the absence of these peaks reliably indicates the absence of the corresponding residue in the unknown sequence. Tryptophan (159 u) also produces a strong immonium ion when it is present in the peptide sequence, but the use of this peak was omitted because it overlaps with other small fragment ions for some peptides. For the automated interpretation of SID spectra, MS-Tag Unknome search parameters were selected as follows: Precursor ion mass tolerance, ±250 ppm for sector-TOF and ±1.0 u for QQ; fragment ion mass tolerance, ±0.3 u for sector-TOF and ±1.0 for QQ. Monoisotopic masses (rather than average masses) were used for both datasets. The fragment ion types expected to be present in SID MS/MS spectra from either instrument included a_i-, b_i-, y_i-type ions, internal sequence ions, and the formal neutral losses of water and ammonia from each of these ion types.

MS-Tag Unknome results for automated peptide sequencing with SID MS/MS spectra acquired separately in the sector-TOF-SID and the QQ-SID instruments are shown in Figure 3. Initial results for the sector-TOF-SID spectra, as indicated by comparison of the dark solid bars (sector-TOF) to the lighter solid bars (dual quadrupole) in Figure 3, did not show higher quality results than QQ-SID spectra for fragmentation of small peptide ions (<700 u), despite the higher mass accuracy available in the sector-TOF data. Closer inspection of the corresponding pairs of SID spectra revealed the presence of fragment ion peaks in the upper regions of the scanned mass range for QQ-SID MS/MS spectra that were indistinguishable from the baseline noise in the sector-TOF spectra. An example of this observation is shown in Figure 4. The measured MS/MS spectrum for singly charged SIGSLAK fragmented by SID activation on a fluorocarbon surface in the sector-TOF instrument contains few fragment ions above *m/z* 350 u clearly distinguishable from the baseline. The corresponding QQ-SID spectrum for this same peptide ion shows multiple lower intensity fragment ions in the mass region between 350 u and 676 u (the precursor ion *m/z* value). Even the higher *m/z* fragments that are present in sector-TOF-SID spectra (such as the b_4 ion in Figure 4) have lower abundances than those measured in the QQ-SID spectra. This fact was consistent for all assayed peptides in the sector-TOF instrument using a fluorocarbon-coated target surface: The "fragment envelope" of the observed ions was shifted to lower masses relative to the QQ instrument. Our group has shown that, for small molecules such as benzene, a 90° collision (such as the one in the sector-TOF instrument) results in a larger percentage of kinetic energy transferred to internal modes of the projectile ion as compared to the 45° collision of the QQ instrument [31]. However, energy deposition as a function of incoming ion beam angle for large peptides during SID has not been extensively studied to date due to the difficulties in theoretically modeling the process and experimentally performing angle-resolved SID.

Excessive fragmentation is not the only factor to be taken into consideration when examining the appearance of higher mass fragments. There are other instrumental factors that may contribute to these features of the sector-TOF SID spectra. The TOF analyzer used in this work uses in-line ion deflection to pulse the ion beam (as opposed to orthogonal acceleration TOFs). In

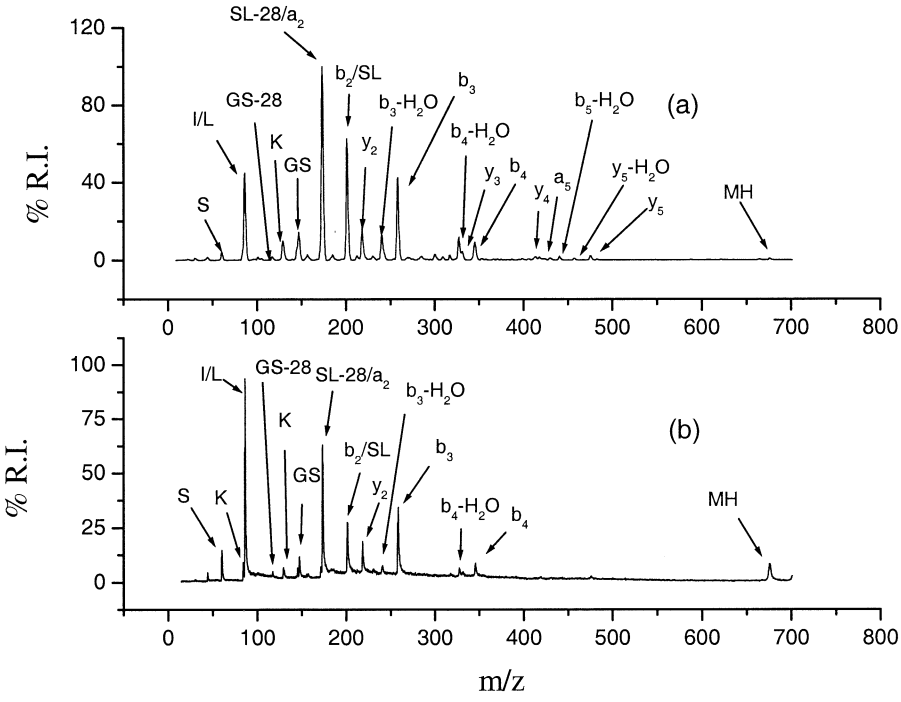

Figure 4. SIGSLAK spectra obtained with **(a)** QQ (45 eV) and **(b)** sector-TOF (45 eV) on a C12F10 surface.

this type of TOF instrument using only 1.25 % of the ions generated in the FAB source are collided against the surface in the sector-TOF (800 ns gate/64 μs maximum flight time). This decrease in duty cycle makes detection of low-abundance high-mass fragments even more difficult. Also, the condition of the microchannel plates in the coaxial detector is a variable that can produce mass discrimination to some extent. The differences in collision geometries in both instruments also might lend to differences in the collection efficiencies for fragment ions not yet completely understood. Experiments using cylindrical (rather than planar) extraction lens geometries at the surface are currently being planned and simulated.

As an alternate way to evaluate the importance of higher mass accuracy and resolution for automated interpretation of SID spectra as a factor independent from the fragmentation extent, the QQ-SID data were truncated to exclude fragment ion *m/z* values higher than the largest sector-TOF-SID fragment ion found and resubmitted to MS-Tag Unknome. These results, indicated in Figure 3 by the striped bars, show that higher-mass-accuracy SID does indeed play a significant role in the quality of the matches obtained by automated sequencing using MS-Tag Unknome. This is evident by the overall decrease in the quality quotient values calculated for the truncated QQ-SID spectra produced from smaller peptides. In some cases, such as for SIGSLAK and DLWQK, the decrease in performance of the sequencing approach with decreasing mass accuracy is so pronounced that absolutely no matches are produced.

As the precursor ion *m/z* value increased, and thus the molecular weight of the unknown peptide increased, the number of possible elemental compositions for that molecular weight increased as well. It is an internal limitation of MS-Tag Unknome that if the input precursor ion mass and the compositional information from immonium ions for a given MS/MS spectrum produce more than 10,000 possible amino acid combinations or more than 500,000,000 possible sequences, an error message will be generated and the Unknome search will not be performed. Automated interpretation of SID MS/MS spectra acquired for larger peptides required an alternative approach.

Peptide Sequencing by SID with a Modified Patchwork Sequencing Approach

Patchwork peptide sequencing was first reported by Schlosser and Lehmann [19] and applied to the interpretation of highly accurate MS/MS spectra ($\pm$10 ppm) generated from peptides produced by tryptic protein digests using collision induced dissociation (CID) in a quadrupole-time-of flight mass spectrometer. In the Patchwork approach, experimental *m/z* values for highly-accurate low-mass fragment ions are matched to calculated accurate mass values for all immonium, y_1, y_2, a_2, and b_2 ions composed of the 20 standard amino acids and assigned accordingly. As a result, very specific information regarding the identities of the amino acid residues occurring at the N- and C-termini of the peptide sequence can be determined and used in the

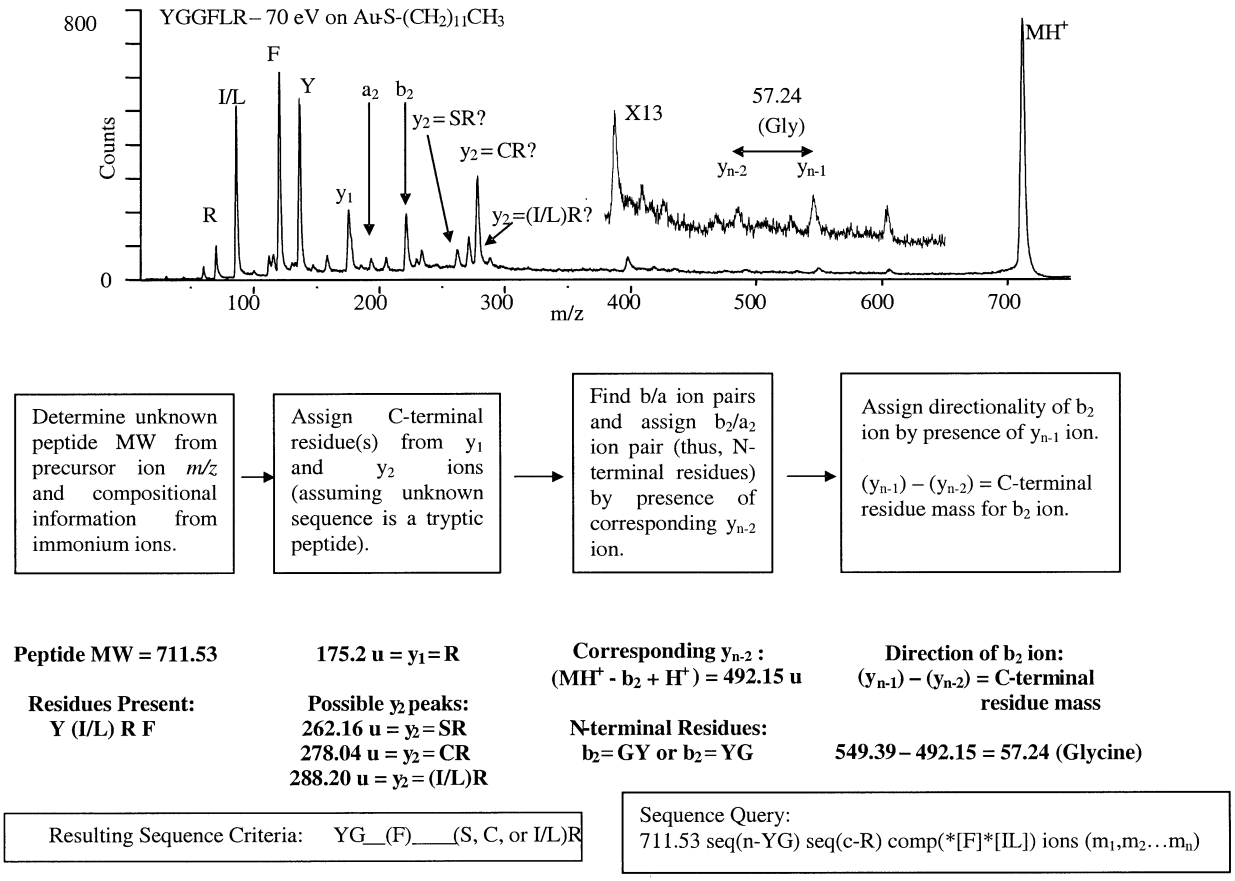

Figure 5. Modified Patchwork sequencing approach for automated interpretation of sector-TOF SID spectra for singly charged YGGFLR fragmented with 70 eV collision on dodecanethiol modified target surface.

generation of candidate peptide sequences. The remaining fragment ions present in the MS/MS spectrum can then be used to confirm the peptide sequence. In our work, a modified version of the Patchwork approach was used. Due to the lower mass accuracy of our TOF-SID setup, two and three-amino acid internal fragment ions could not be identified with sufficient accuracy and thus instead of sequence qualifiers for these internal ions, compositional qualifiers together with C-terminal and N-terminal sequence qualifiers were used as query filters for MASCOT. The internal ions intensities are only incorporated in the sequence search using the "ions(m_1,m_2,m_3,m_n)" command available in Mascot. We refer to this procedure as the "modified" Patchwork approach

The basic steps involved in our version of Patchwork sequencing are presented in Figure 5, where YGGFLR is used as an example. Initially, a molecular weight for the unknown peptide sequence is calculated from the singly charged precursor ion *m/z* ratio. Compositional information from the presence of immonium ions in the spectrum is then noted. This results in an unknown peptide molecular weight of 711.53 g mol^{-1} and the presence of tyrosine (Y), isoleucine or leucine (I/L), arginine (R), and phenylalanine (F) in the example shown in Figure 5. (Certainty in the presence of these residues also limits the remaining potential amino acids candidates to the ones that would match the *m/z* difference between the precursor ion and the immonium ions). Additional spectral information can then be used to assign the identity of the residues located at the C-terminus. Assuming the unknown peptide sequence is a tryptic digestion product, the C-terminal residue can be identified by the presence of the y_1 ion as either *m/z* 147.11 or *m/z* 175.11, corresponding to C-terminal arginine (R) or lysine (K), respectively. Within the fragment mass tolerance of the sector-TOF-SID data (±0.3 u), the y_1 = K ion overlaps with a_2 = AC and a_2 = SS ions, however the presence of a corresponding b_2 ion for each of these a_2 ions (equivalent to a_2 plus the exact mass for carbon monoxide: 27.99436605 u) would indicate the true identity of the peak at *m/z* 147.11 as an a_2 ion rather than the y_1 = K ion. The y_1 = R ion overlaps with b_2 = AC and b_2 = SS ions and likewise, the presence of the corresponding a_2 ions can flag a misidentified y_1 = R ion as well. In the event that the unknown peptide sequence contained AC or SS as the N-terminal residues and R as the C-terminal residue, the MS/MS spectrum is likely to contain both *m/z* 147.11 and 175.11; in that case, no C-terminal residue

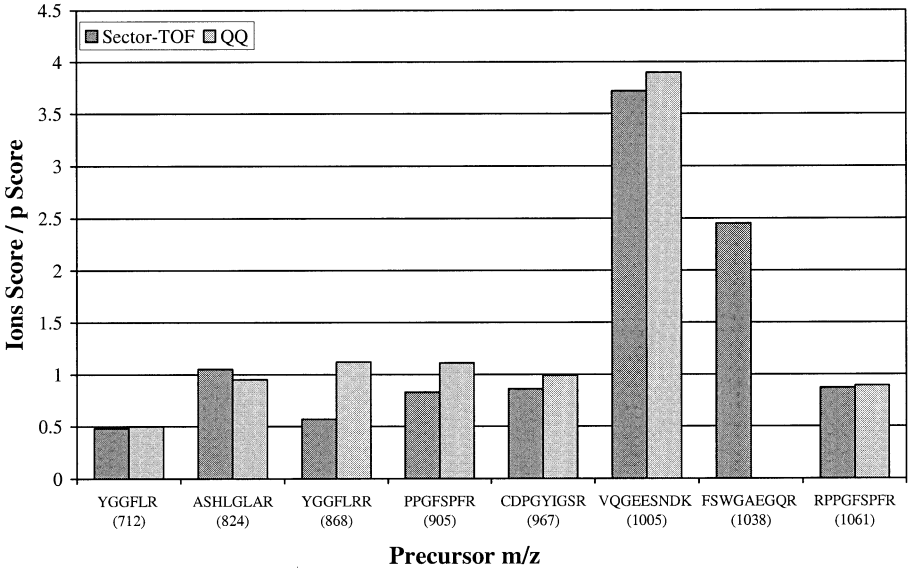

Figure 6. Patchwork results (no enzyme specified) for larger peptides (>700 u) from sector-TOF-SID (+/− 0.3 u; dark solid bars) and dual quadrupole-SID (+/− 1.0 u; light solid bars) using a C12F10-modified collision surface. An ions score to *p* score ratio larger than 1 represents a successful identification of the peptide sequence.

information could be confidently assigned for sector-TOF-SID data. The potential y_2 ion is then identified by the appropriate set of dipeptide combinations that contain the previously identified y_1 ion residue. From the example spectrum in Figure 5, these two steps revealed that the potential C-terminal residues are (S, C, or I/L) and R. N-terminal residues are assigned in the following manner: All fragment ion peaks separated by the mass difference of a neutral loss of carbon monoxide are assumed to be b_i/a_i ion pairs. For MS/MS spectra with mass accuracy greater than or equal to ±10 ppm, combinations of two and three amino acid residues with total protonated masses equal to the ones of suspected b_2, b_3 or internal ions can be directly assigned [19]. However, with the lower mass accuracy of the sector-TOF-SID spectra, sequencing information can only be derived from the b_2/a_2 ion pair (or the b_2-17/a_2-17 pair if present). The b_2/a_2 ion pair was assigned by the presence of the corresponding y_{n-2} ion in the SID spectrum (n = number of residues). Subtracting the b_2 ion *m/z* value from the precursor ion *m/z* value and adding the exact mass of an ionizing proton (1.007825035 u) allows the expected *m/z* value of the y_{n-2} ion to be calculated. For the example spectrum in Figure 5, *m/z* 492.15 is present, indicating a b_i/a_i ion pair at *m/z* 221.09 and 193.09 is the b_2/a_2 ion pair. The N-terminal residues can then be assigned for the unknown peptide sequence as GY or YG. The directionality of the b_2 ion can then be determined by the presence of the y_{n-1} ion. The mass difference between the fragment ion peaks for y_n-1 and y_n-2 will be equivalent to the mass of the C-terminal residue in the b_2 ion. The mass difference in the example (for *m/z* 549.39 and *m/z* 492.15) is equal to 57.24 u, identifying glycine as the C-terminal residue in the b_2 ion and allowing the unambiguous assignment of YG as the unidirectional N-terminal residues. We can then conclude that the unknown peptide sequence for which the SID spectrum is shown in Figure 5 must contain YG as the N-terminal residues, S, C, I, or L coupled with R as the C-terminal residues and also that F must be present in the remaining internal portion of the sequence. These very specific sequence criteria were then used to search for the correct peptide sequence within a protein database (SwissProt) with Mascot's [18] Sequence Query. The fundamental approach in Mascot scoring of candidate peptide sequences is to calculate the probability that the observed match between the experimental data set and each sequence database entry is a chance event. The peptide sequence match with the lowest probability will have the largest "Ions Score" (sometimes also referred to as MOWSE scores). Mascot output results for peptide sequence matches are given in the form of an Ions Score for each candidate sequence together with an "identity" score that can be used as a cutoff score to exclude random matches. The cutoff score (referred to as "p Score" in Figure 6) sets a probability limit ($P > 0.95$) above which a peptide must score to be considered a significant match. Peptides sequences with Ions Score at or below the p Score limit have a higher likelihood of being a random match to the MS/MS data (i.e., a false positive).

Figure 6 shows sequence database search results using the modified Patchwork approach for sector-TOF-SID and QQ-SID MS/MS spectra. The ratio of the Ions Score for the correct peptide sequence to the p Score for the corresponding spectrum for larger peptides (>700 u) collided with a fluorinated SAM

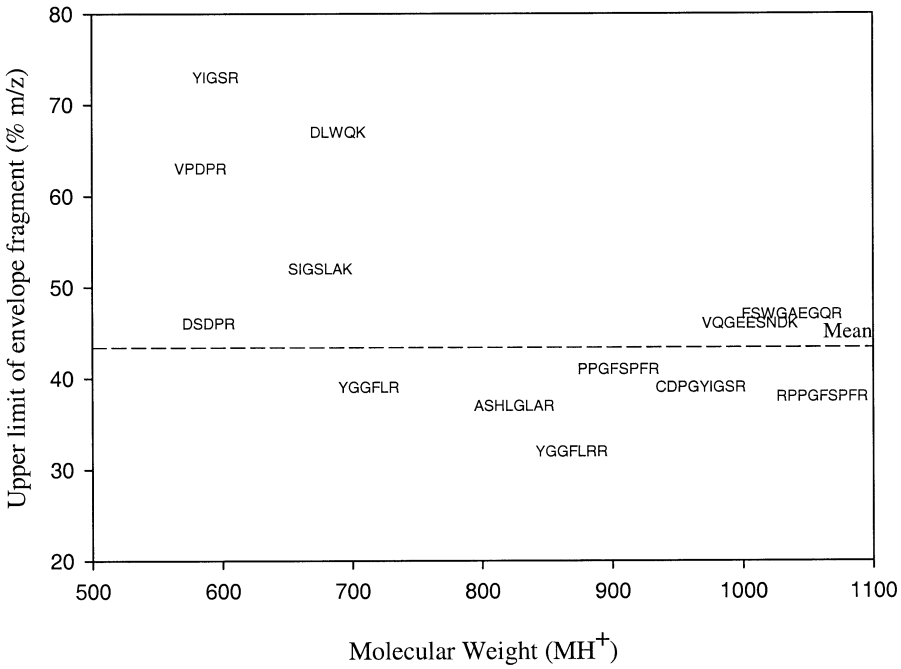

Figure 7. Mass to charge value of largest identified ion in the fragment ion spectral envelope versus the precursor ion molecular weight for 45 eV collisions in the sector-TOF instrument. The upper limit of the fragment ion envelope is expressed as a percentage of the precursor ion molecular weight. A lower value in the ordinate means a larger gap between the largest fragment in the envelope and the precursor ion *m/z* value.

(C12F10) is plotted. A comparison of sector-TOF results (dark bars) to QQ results (light bars) shows that two immediate conclusions might be drawn: First, most of the SID spectra do not score significantly above the Ions Score to p Score cutoff value (ratio lower than one), regardless of the instrument from which they were acquired (and thus, the extent of fragmentation observed). Second, there seems to be no apparent advantage for the use of sector-TOF-SID spectra with higher mass accuracy and resolution in this database search approach. Similarly to the smaller peptides, closer comparison of sector-TOF spectra with the corresponding QQ spectra reveals that the envelope of fragment ions extends from low *m/z* values to mid *m/z* values, ending at much lower *m/z* values in the sector-TOF spectra for all the larger peptides examined in this dataset. Figure 7 shows the relationship between the largest fragment ion present in the sector-TOF spectrum with respect to the precursor ion *m/z* value (% *m/z*) and the singly charged precursor peptide ion *m/z* value for peptides ranging from 583.36 to 1060.57 u. Peptides that scored well for both MS-Tag Unknome (Figure 3) and the modified Patchwork/Sequence Query approach (data not shown) had precursor ion *m/z* values less than 700 u and the respective fragmentation envelope within these spectra extended between ~45 and 73% of the scanned mass range. Sector-TOF spectra for larger peptides (>700 u) scored poorly for the Patchwork/Sequence Query approach. These larger peptides have a significantly narrower envelope for fragment ion *m/z* values than that of the smaller peptide ions, ranging between ~31 and 47% of the scanned mass range. Low mass fragment ions are abundant in the MS/MS spectrum, but the mass range over which the fragment ions extend is quite narrow with respect to the scanned mass range. The increased lack of an extended fragment mass range masks any possible effect that enhanced mass accuracy might have on the results. In an attempt to improve ion collection after collision with the surface so as to extend the mass window for the fragments, the composition of the target surface was altered. Less stiff surfaces with lower *m/z* tail groups composed of pure hydrocarbon and mixed hydrocarbon/fluorocarbon SAMs [31] were used rather than the pure fluorocarbon surface.

Surface-Induced Dissociation on Pure Hydrocarbon and Mixed Fluorocarbon/Hydrocarbon Monolayers

As previously observed, high mass accuracy measurements improved successful peptide sequencing using SID MS/MS data. Indeed, the results obtained for the QQ instrument where a continuous ion series is present suggest that obtaining that same series of fragment ions in the TOF instrument with high mass accuracy should be beneficial. In an effort to obtain higher mass accuracy mass spectra with a larger range of fragment ion masses present for larger peptides (>700 u), mixed monolayers

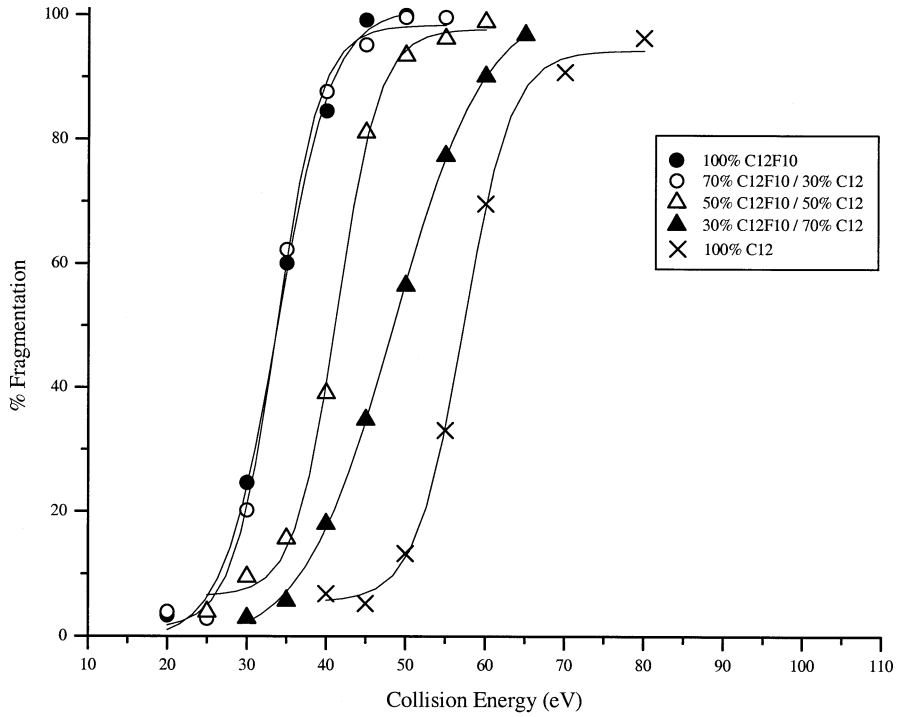

Figure 8. Fragmentation efficiency curves generated by collision of singly charged YGGFLR with a variety of pure and mixed composition SAM target surfaces using the QQ instrument.

self-assembled from ethanolic solution mixtures of C12F10 and C12 and single component C12 solutions were used as collision targets.

Recent work by Futrell and coworkers has shown that when singly protonated des-Arg^1-bradykinin is used as a projectile, an H-SAM assembled from 1-dodecanethiol showed an energy transfer efficiency of 10.1% while for its semifluorinated analog ($CF_3(CF_2)_9C_2H_4SH$) this efficiency increased to 20.5% [32]. These authors concluded that the increase in the average energy deposited into the ion is a function of the increase in mass of the chemical moiety that the projectile ion encounters upon collision, whereas the width of the energy deposition function is one of surface stiffness. In their experiments, Futrell and coworkers showed also that, after collision, both H-SAM and F-SAM surfaces produced internal energy distributions in the projectile ion (PPGFSPFR) of similar width. Hase and coworkers have simulated collisions of $Cr(CO)_6^+$ with diamond surfaces and hexanethiolate H-SAMs and proposed that the internal energy gained by the projectile ion is strongly dependent on the target surface stiffness [33–35]. Atomic force microscopy results have shown that the replacement of the stiffer fluorocarbon chains by more flexible and lighter hydrocarbon chains changes the overall hardness of the assembled monolayer and the packing of the thiol chains [36, 37] and thus it is expected that the simultaneous change in rigidity and mass of the collision partner at the surface will change the intrinsic characteristics of the energy transfer process [35]. The different packing angle [36] of the fluorinated chains (16°) versus the hydrocarbon chains (27°) might also possibly change the geometry of the scattered fragment ions plume, thus producing different ion collection by the ion optics. Molecular beam scattering experiments have also shown that the chemical nature of the SAM surface influences the velocity of the ions scattered from the surface due to the energy transfer to low frequency modes of the surface [38]. The mixed monolayers were assembled from thiols of similar chain length in order to ensure a uniform SAM assembly [39].

Figure 8 shows the percent of YGGFLR fragmentation when different pure and mixed monolayers are used as collision targets in the QQ instrument (45° collision). Clearly, the use of a less stiff surface with lower *m/z* terminal groups shifts the onset collision energy of the sigmoid-like curve to larger values. This general trend was also observed in the sector-TOF instrument, but with an interesting difference not observed in the QQ instrument: the relative abundance of larger mass fragments increases when hydrocarbon surfaces are used. Figure 9 shows this effect when the singly charged peptide LMYPTYLK is collided with three different surfaces: C12, C12F10, and a 50:50 solution mixture of both thiols. As can be seen in Figure 9, the 50:50 mixed monolayer only provides a shift in the energy scale. However, a hydrocarbon monolayer not only shows a shift to lower internal energy deposition, but also an increase in the relative abundance of heavier fragments, up to a 100% increase in the relative intensity for the y_6 fragment ion (Figure 9, bottom right). The

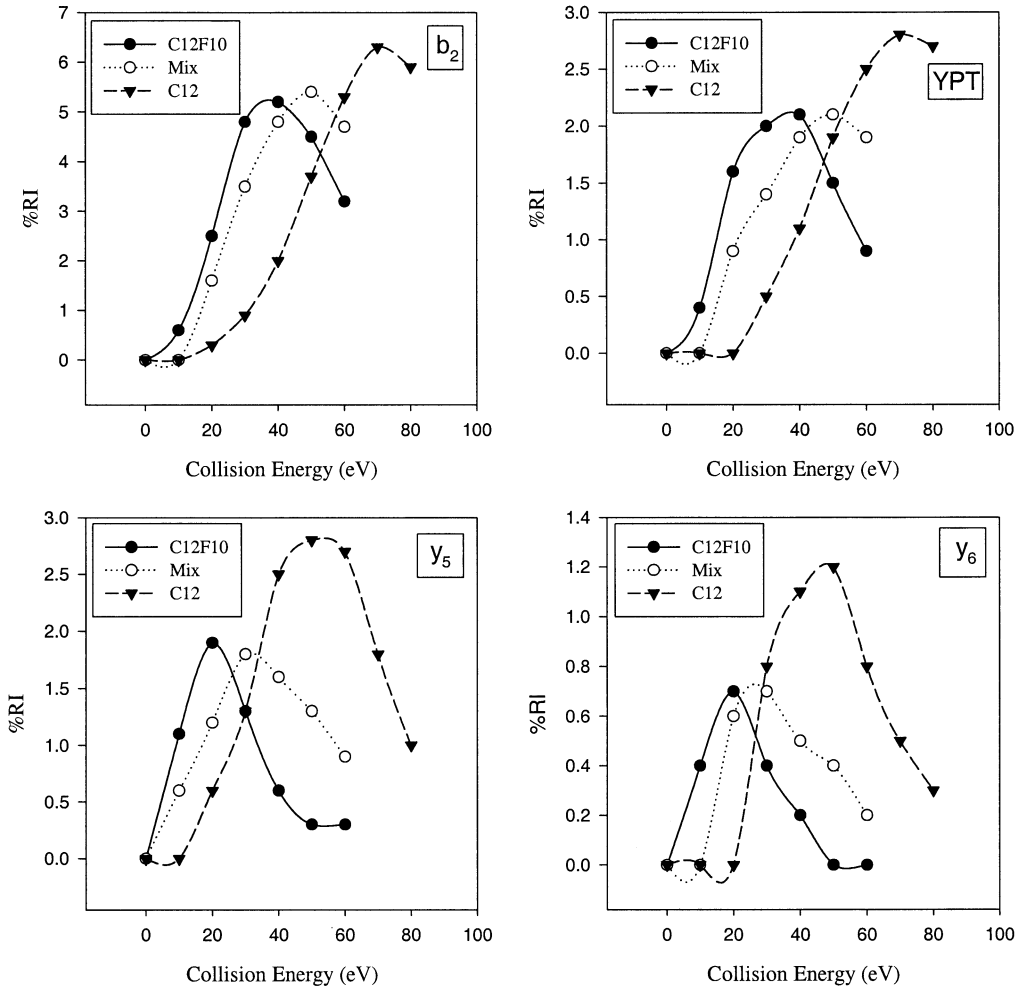

Figure 9. Abundance of different fragments produced by SID fragmentation of singly-charged LMYPTYLK collided with pure C12, C12F10 and mixed (50:50) SAM films using the sector-TOF instrument: (top left) b_2 fragment, (top right) YPT fragment, (bottom left) y_5 fragment, (bottom right) y_6 fragment.

increase in relative intensity loosely follows the increase in fragment ion mass, indicating that there is a quantitative improvement in the ion collection process due to the change in the chemical nature of the collision surface. For a small fragment, the remaining kinetic energy after collision is low enough as to ensure efficient collection in the region encompassing the target surface and the last reflectron plate. Larger fragments will retain larger kinetic energy after collision, which can be lowered by using a less stiff surface (C12 rather than C12F10) [38], thus facilitating collection. The increase in intensity of larger mass fragments when using pure C12 hydrocarbon surfaces in combination with TOF mass analysis should then be beneficial for sequencing purposes.

Figure 10 shows the modified Patchwork sequencing results of peptides activated by SID on a C12 surface using both instruments. Larger laboratory collision energies were used because of the lower internal energy deposition provided by the H-SAM. Different collision energies were chosen for every peptide in order to maximize the number of fragmentations but maintained constant in both instruments. In practical applications, collision energy would be stepped or ramped. In general, the fragmentation efficiency during SID experiments on H-SAMs seemed to be more sensitive to the composition and molecular weight of the projectile ion than during SID experiments on F-SAMS. Collision energies were as follows: YIGSR: 55 eV, YGGFLR: 65 eV, ASHLGLAR: 75 eV, PPGFSPFR: 65 eV, CDPGYIGSR: 65 eV, RPPGFSPFR: 95 eV. As expected from the results shown in Figure 9, there is a clear improvement in the Ions Score to p Score ratio for all the assayed peptides when a C12 rather than a C12F10 surface is used in the TOF instrument. The Ions Score to p Score ratios for the TOF-SID instrument using a C12 surface shown in Figure 10 are better than those for the QQ instrument using either a C12 or a C12F10 surface (see Figure 6), showing the combined effect of enhanced fragment ion collection and mass accuracy. In the case

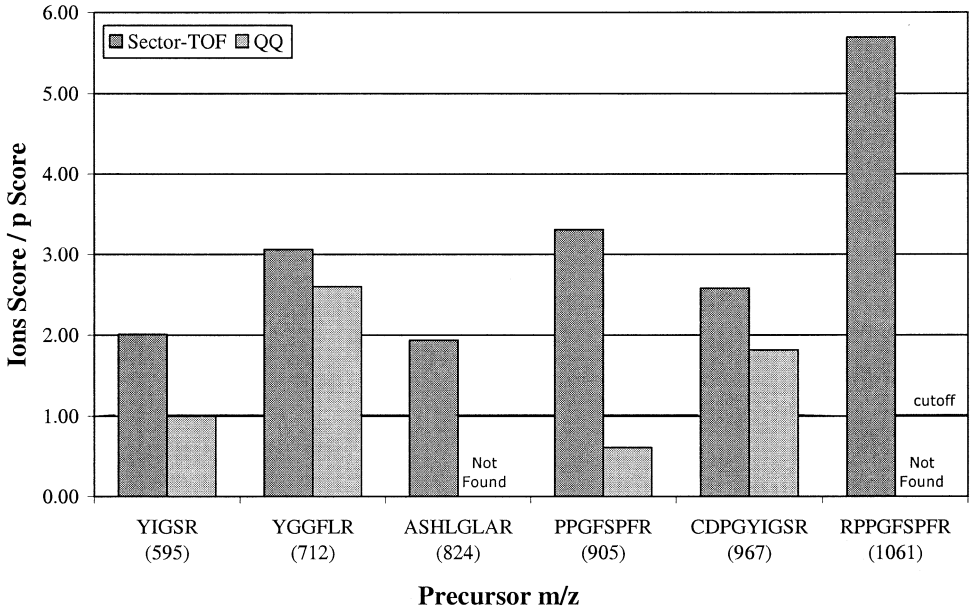

Figure 10. Simplified Patchwork results (no enzyme specified) for larger peptides (>700 u) using a C12 SAM as collision surface from sector-TOF-SID (dark solid bars), and from dual quadrupole-SID (light solid bars). The correct sequences for ASHLGLAR and RPPGFSPFR were not found by Sequence Query. Collision energies were as follows: YIGSR: 55 eV, YGGFLR: 65 eV, ASHLGLAR: 75 eV, PPGFSPFR: 65 eV, CDPGYIGSR: 65 eV, RPPGFSPFR: 95 eV.

of the QQ-SID instrument using a C12 surface (Figure 10, light bars), some of the assayed peptides (ASHLGLAR, PPGFSPFR, RPPGFSPFR) that showed Ions Score to p Score ratio larger than zero in Figure 6 could not be found. This result can be attributed to incomplete fragmentation of the peptides due to the combined effect of a 45 degree collision angle in the QQ instrument and a collision target with lower effective mass, which translates into lower energy deposition, as can be seen in Figure 11. For peptides such as RPPGFSPFR (MW_{MH} = 1061.23 u), ASHLGLAR (MW_{MH} = 824.47 u) and PPGFSPFR (MW_{MH} = 904.47 u) the correct peptide sequences were not found by the database search algorithm due to the fact that in these particular cases, fragment ions that are crucial for narrowing down the number of candidate sequences during Patchwork sequencing could not be unequivocally assigned. The b_2 fragment ion could not be assigned in the spectra obtained for RPPGFSPFR and ASHLGLAR and the y_2 fragment ion could not be assigned in the PPGFSPFR spectra, probably due to incomplete fragmentation, even at high collision energies (up to 95 eV was used for RPPGFSPFR and 75 eV for ASHLGLAR). Larger collision energies were not assayed. The absence of the b_2 and/or y_2 fragment ions limits the number of sequence qualifiers that can be extracted from the spectrum using the approach described on Figure 5. Only composition qualifiers (in addition to the M_w and ions list) could be used for RPPGFSPFR. For ASHLGLAR, only C-terminal, y_1 sequence information could be assigned. Again, the lack of information about the b_2 ion was critical in not being able to identify the correct peptide sequence. In the case of PPGFSPFR, although the y_2 fragment ion could not be found (only y_1 was assigned), the right sequence was found within the list of potential sequences due to the fact that the b_2 fragment ion could be assigned. Clearly, this database search approach is more sensitive to the lack of N-terminal sequence information (absence of b_2) than to the lack of C-terminal information (absence of y_2). The number of possible amino acid sequences for a tryptic peptide is smaller at the C-terminus than at the N-terminus, due to the constraint that the C-terminal residue has to be R or K.

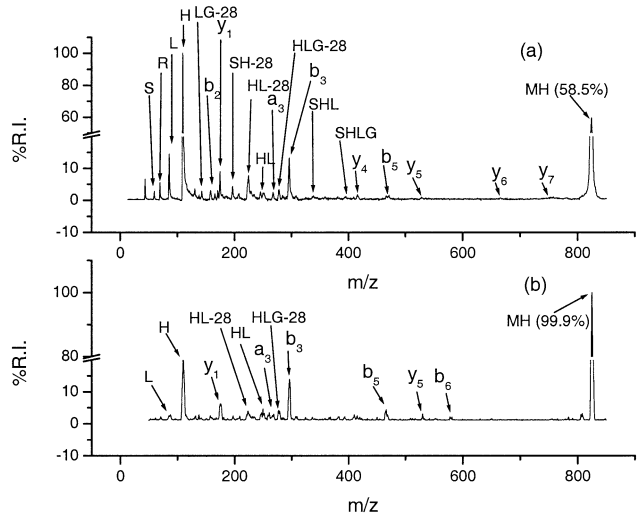

Figure 11. ASHLGLAR spectra obtained with (**a**) sector-TOF (75 eV) and (**b**) QQ (75 eV) on a C12 surface. Notice that the y-axis break points are set at different positions in each case.

Conclusions

We have demonstrated that an alternative sequencing approach based on the "Patchwork principle" with unconventional SID MS/MS data is feasible. As expected, accurate mass analysis of the resulting fragment ions is a highly desirable quality in the design of any new SID mass spectrometer. Possibly, the use of orthogonal acceleration-TOF [40] instead of the current linear-modulation TOF would allow an increased duty cycle with a simultaneous increase in mass accuracy. Hydrocarbon monolayers, rather than fluorocarbon ones have been shown to be more suitable for sequencing applications with the presently available TOF instrument. It is also clear that the existing database search algorithms are not specifically designed for SID MS/MS spectral data and thus their unconventional use currently requires a large amount of user intervention. Automation of the Patchwork approach and development of improved algorithms that incorporate knowledge about gas-phase peptide ion chemistry in SID processes is needed.

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