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Tandem Time-of-Flight (TOF/TOF) Mass Spectrometry and Proteomics†

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Abstract

A dual reflectron *tandem time-of-flight* (TOF/TOF) mass spectrometer reported in 1993 gave rise to the invention and development of the *curved-field reflectron* (CFR) for focusing product ions. The CFR is used in this case as the second mass analyzer in a tandem instrument (based on the Kratos AXIMA CFR) in which the first mass analyzer is a linear TOF that focuses ions by pulsed extraction. Because ions can be focused over a broad range of kinetic energies, deceleration of precursor ions and/or reacceleration of product ions is not required. Thus, product ions produced by post-source processes (*laser induced dissociation* or LID, metastable decomposition and opportunistic collisions) are recorded in the product ion mass spectra at the same times as their isomass ions produced by *collision induced dissociation* (CID). In general both LID and CID product ion mass spectra are very similar, producing primarily *b*-series and *y*-series ions, though there is some preference for fragmentation at weaker bonds such as those at proline or aspartic acid residues. The tandem mass spectrometer has been used to determine the acetylation sites for a *histone acetyl transferase* (HAT) protein. A novel and improved method for derivatizing tryptic fragments by N-terminal sulfonation produces almost exclusively *y*-series ions, and has been used to determine protein ubiquitination. The tandem mass spectrometer has also been used to identify potential biomarkers associated with heart failure, in particular that fraction containing albumin that is generally removed from serum samples to permit protein biomarker analysis. Analysis of the unfractionated serum, the *albuminome*, and the depleted serum is also carried out using *surface-enhanced laser desorption/ionization* (SELDI) and the high molecular weight proteins are monitored by using a Comet macromizer™ TOF mass spectrometer with a very high mass cryocooled detector.

Keywords

Curved-field reflectron; Laser induced dissociation; Opportunistic collisions; Histone acetyl transferase; Surface-enhanced laser desorption/ionization

1. INTRODUCTION

In 1993 our laboratory introduced a tandem time-of-flight (TOF/TOF) mass spectrometer (Fig. 1) with two reflectron mass analyzers separated by a mass-selector gate and collision region into which a collision gas was pulsed once each mass recording cycle. The source, mass analyzers, and collision region were constructed in such a way as to permit all linear, reflecting and the collision regions to be floated so that ions could be decelerated and a

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range of collision energies (in the laboratory frame) could be used. While the first instrument used two *dual-stage* reflectrons,¹⁾ a later version with two *single-stage* reflectrons provided the opportunity to record a wider range of product ion masses on a linear mass scale.²⁾

The ability to decelerate the precursor ions and then reaccelerate the product ions also improves focusing by the reflectron. Product ions carry only a portion of the kinetic energy, *i.e.*, $E_2 = (m_2/m_1)E_1$, where m_1 and m_2 are the masses of the precursor and product ions, respectively and E_1 is the kinetic energy (in the laboratory frame) of the precursor ion. The reflectron has a limited bandwidth that cannot accommodate the entire range of energies, in this case from 0 to 5 keV as determined by the ion source accelerating voltage of 5 kV. However, by decelerating the ions to 1 keV prior to collision and then reaccelerating the product ions by 4 keV, then the kinetic energy range of the product ions is from 4–5 keV. This approach was used successfully by Enke *et al.*³⁾ in a two reflectron tandem mass spectrometer in which precursor ions were fragmented by photodissociation. In our case, however, reduction of the kinetic energy E_1 also reduces the available collision energy.

Thus, the *curved-field reflectron* (CFR) was developed to enable us to utilize the full kinetic energy, resulting from the initial ion acceleration, as the collision energy.⁴⁾ The CFR, while developed for the tandem instrument, was also very effective for obtaining *post-source decay* (PSD) product ion mass spectra on a single reflectron instrument.^{5), 6)} PSD was introduced by Spengler *et al.*⁷⁾ for single mass analyzer instruments with a reflectron, but required changing the reflectron voltage to focus separately different regions of the product ion mass spectrum. The CFR, however, enabled recording of the entire product ion mass range at a single, fixed voltage.

2. Results and Discussion

2.1 Tandem mass spectrometers using pulsed extraction and a single reflectron

One of the disadvantages of the two reflectron instrument was that considerable post-source decay occurred before the ions entered the first reflectron. The resulting product ions do not arrive at the collision chamber and mass-selector at the same time as their precursor, and so are gated out from the spectrum. Thus, a considerably more sensible approach would be to use a simple, linear TOF as the first mass analyzer, using the fact that precursors and their products will maintain the same velocity and reach the mass-selector at the same time. The combination of a pulsed, linear TOF as a first mass analyzer and a reflectron second mass analyzer is the approach generally used in current tandem TOF instruments, though in some cases deceleration of the ions prior to reaching the collision chamber loses the advantage of recording *all* mass-selected post source product ions.

For example, in the instrument shown in Fig. 2a and described by Medzihradszky *et al.*⁸⁾ ions formed in the source are accelerated to energies of 20 keV, mass-selected by a *timed ion gate*, and then decelerated to 1–2 keV before entering the collision chamber. After the ions are passed through the collision chamber, the remaining precursor and the product ions are reaccelerated into the second, reflectron mass analyzer using a delayed extraction pulse. In effect this is a second source, adding 18 keV to the kinetic energies of all ions, so that the resultant band of energies entering a reflectron optimized for 20 keV ions is in the focusable range of 18–20 keV. Figure 2b is a representation of the kinetic energies of the ions during the course of the experiment, showing the deceleration of the ions before the source and their reacceleration into the second mass analyzer. If the precursor ion has an m/z of 1000, then all product ions formed *before* the collision with m/z values below 900 will have kinetic energies less than 18 keV and will be reflected by the decelerating field and will not enter the collision chamber. Those product ions above m/z 900 will enter the collision chamber at

different times. In either case these *early PSD* ions will not be recorded in the product ion mass spectra. Product ions formed after reacceleration will enter the reflectron, but at different times than ions of the same mass that were formed in the collision chamber. Thus, these *late PSD* ions appear as artifact in the mass spectrum, and are generally removed by gating or deflection.

In the instrument shown in Fig. 3a, ions are formed in the ion source, accelerated to 8 keV and enter the collision chamber with this energy. 9) Product ions formed prior to entrance to the collision chamber will have the same velocities as their mass-selected precursor, whether this occurs before or after the mass selection gate. Thus, these *early PSD* ions will have the same flight times as those produced by CID and will contribute to the focused ion signal at the detector. The set of product ions from a mass-selected precursor, along with their precursor, enter a *lift cell*, a field free region whose voltage can be lifted while the ions are in residence and provide additional acceleration energy when they are extracted into the second mass analyzer. Product ions formed after this time, that is: *late PSD* ions, would arrive at different times than their counterparts formed in the collision chamber or before. A second timing gate can be used to remove the remaining precursor ions (which come out later than the CID products) as well as any new product ions that are formed. These two approaches are used on tandem instruments developed for commercial use by Applied BioSystems (Foster City, CA) and Bruker Daltonics (Bremen, Germany), respectively and both provide the means for focusing product ions without changing the reflectron voltage. Because the latter approach focuses product ions formed before or in the collision chamber in the same way, this approach is also being used for PSD instruments as well.

We recently modified a Kratos (Manchester, UK) AXIMA CFR mass spectrometer by the addition of a collision chamber between the ionization source and the mass-selection gate. 10) As shown in Fig. 4a, precursor ions are accelerated to an energy of 20 keV and maintain that energy (in the laboratory frame) as they are injected into the collision chamber. As shown in Fig. 4b, the ions are not decelerated or accelerated at any time between leaving the ionization source and entering the reflectron. Thus, product ions formed before or after the collision chamber and/or mass selection gate will maintain the same velocities as their corresponding precursors and will only be differentiated in time as they are transmitted through the reflectron. While this scheme is possible using a simple single-stage reflectron, the use of the *curved-field reflectron* (CFR) in this case provides focusing across the product ion mass and energy range.

2.2 Post-source decay, laser-induced dissociation and CID

In our instrument the collision chamber is a cylinder 1.13 in long and 0.2 in i.d. The collision gas (generally helium) is admitted in the center of the cylinder through a long (2 meter) 0.070 mm i.d. glass capillary tube at a flow rate of 0–1 mL/min. The limited gas conductance of the cylinder makes it possible to achieve relatively high pressure in the center of the collision region, while maintaining high vacuum in the surrounding chamber. However, it is not possible to measure the pressure in the cylinder, so that the backing pressure at the gas cylinder regulator and the vacuum in the mass analyzer is used to provide reproducible or comparative collision conditions. The ability to carry out effective CID in this instrument is shown in Fig. 5, in which the product ions from buckminsterfullerene (C_{60}) are shown as a function of the helium gas pressure. In Fig. 5a, ions with m/z 720 are selected, but there is no helium gas and no fragmentation is observed. At 20 and 40 psi (Figs. 5b and 5c), fragment ions with even numbers of carbons, C_{2n} , are observed down to C_{32} . CID mass spectra at higher pressures in Figs. 5d to 5f show the characteristic “catastrophic fragmentation” as the remaining half of the molecule dissociates into C_n fragments, as well as “magic numbers” at C_{11} , C_{15} , C_{19} and C_{23} . This fragmentation pattern has been observed for fullerenes ionized by *fast atom bombardment* (FAB) on tandem sector

instruments,¹¹) as well as on our previous tandem TOF mass spectrometer.¹²) From these previous reports it is known that attenuation of the precursor ion beam at these higher helium pressures is very high, from 80% to 98%, and that the appearance of the lower mass fragments coincides with a high occurrence of multiple collisions. Figure 6 shows the attenuation of the C₆₀ ion (including both fragmentation and scattering) as a function of helium gas pressure. The C₅₀ fragment ion (at m/z 600) reaches a maximum around 2×10^{-5} Torr vacuum (or 50 psi at the gas cylinder), while the C₁₅ ion continues to increase in part (presumably) from secondary collisions of the higher mass fragments.

The fragmentation of fullerene as shown here is primarily a CID process, with very little fragmentation observed when there is no gas to provide collisions that will activate the molecular ion. It is also clearly a high energy collision process, with fragmentation occurring with one or only a few collisions. For peptide ions formed by MALDI, fragmentation is observed easily without the addition of a collision gas by the method known as *post-source decay* (PSD), in which the processes for activation may include the ionization process, metastable fragmentation and opportunistic collisions with residual gas. In tandem time-of-flight (TOF/TOF) instruments it is common to record product ion mass spectra without any collision gas, which is essentially the same processes, but the method is generally termed *laser-induced dissociation* (LID) to distinguish it from methods in which the reflectron voltage was stepped. In addition, the ions observed in the high energy CID mass spectra when collision gas is introduced to the tandem TOF instruments is not very different from that observed by PSD (or LID). As shown in Fig. 7, the major effect of increasing the collision gas pressure is an increase in the lower mass fragments.

2.3 Tandem TOF mass spectra of peptides

Figure 8 shows the LID tandem mass spectra of two peptides from the tryptic digest of *histone acetyl transferase* (HAT). Even in the absence of a collision gas both peptides show nearly complete *b*-series and *y*-series sequence ions. Figure 9 is the MS/MS spectrum of another tryptic peptide from the HAT protein that contains two proline residues. The *y*-series ions show preferential cleavage of the amide bond on the amino side of the proline residues, which is generally considered to be the weakest bond. At the same time, there are complete *b*-series and *y*-series ions in the region recorded here. We note here that one of the motivations for the recent development of *electron capture dissociation* (ECD) has been its ability to promote fragmentation more randomly rather than at the weakest bond.¹³, 14) Fragmentation of the weakest bond is often cited as one of the drawbacks of CID, but in fact it is primarily a property of low energy CID in which the molecule is activated by repeated or multiple collisions. Similar results are obtained using thermal methods of activation, such as *infrared multiphoton dissociation* or IRMPD.¹³) It should also be noted that a property of high energy CID as first described by Gross *et al.*¹⁵) is that fragmentation is more randomly distributed and may be remote from the location of the charge site. In addition, this *charge-remote fragmentation* is also observed for PSD.¹⁶) Thus, the fragmentation shown here, and originating from excitation during the laser desorption process, appears to have elements of both low and high energy CID.

Figure 10 is the MS/MS spectrum of a tryptic peptide from the HAT protein with two acetylated lysine residues.¹⁷, 18) The prominent peaks labeled *b*₁₂ and *b*₁₇ occur at aspartate residues. While these may represent the weakest bonds, there are a number of *a*-, *b*-, and *c*-series ions covering much of the amino acid sequence. We have recently described a protocol for N-terminal sulfonation of tryptic peptides¹⁹) that greatly simplifies the MS/MS spectra by generating exclusively *y*-series ions. The chemical derivatization occurs at very high efficiency, and therefore can improve the overall signal/noise considerably. Figure 11a shows the MS/MS spectrum of the peptide LGIHEDSQNR sulfonated at the N-terminus. In addition to the complete set of *y*-series ions, the spectrum also shows a

prominent and characteristic loss of the sulfonation tag ($\text{HO}_3\text{S}-\text{C}_6\text{H}_4-\text{NCS}$, 215 Da). This method is being used currently to determine ubiquitination sites on proteins. Ubiquitin attaches to lysine residues in proteins through its C-terminal -RGG sequence. When ubiquitinated proteins are digested with trypsin, cleavage at this arginine leaves a GG tag on the ubiquitination site. When the resulting ubiquitinated tryptic peptides are derivatized, there are two N-terminal sulfonation sites, on the peptide itself and on the GG branch. Figure 11b shows the MS/MS spectrum of a tryptic peptide Ub_{43-54} obtained from tetra-ubiquitin. The region of the spectrum that provides the signature for ubiquitination includes peaks corresponding to the loss of one or two sulfonation tags, with one or more of the residues (G or L) adjacent to each of these sulfonated groups. The MS/MS spectrum also provides a complete set of γ -series ions that determine the amino acid sequence and reveal the ubiquitination site.²⁰⁾

2.4 The “albuminome”

A number of mass spectral techniques are currently being used for the discovery and identification of biomarkers in serum for heart failure, myocardial infarction and vasculitis. 21) Analysis of proteins from whole serum is generally not practical since serum is dominated by several large, very abundant proteins, particularly albumin and IgG. Removal of albumin in fact helps to reveal many more proteins as is shown in Fig. 12, comparing the mass spectra of whole serum and albumin-depleted serum using a Ciphergen (Fremont, CA) CM-10 SELDI chip in the AXIMA mass spectrometer. Our procedure involves the extraction of proteins from serum in 0.1 MNaCl, addition of Protein G affinity resin to remove IgG, extraction in 42% ethanol for 1 hour at 4°C and ultracentrifugation at 16,000 *g*. The albumin depleted serum is recovered in the pellet, while the fraction containing albumin is found in the supernatant. While it is common to monitor such separations using SDS-PAGE, we have more recently used a Comet (Flammatt, Switzerland) macromizer™ mass spectrometer with a cryocooled detector for recording very high mass proteins. The macromizer mass spectra of the depleted and albumin fractions are shown in Fig. 13.

The albumin fraction contains many proteins in addition to albumin and therefore may itself be a source of biomarkers. For this reason it can be termed the *albuminome*. When denaturing conditions are applied the supernatant, this fraction yields a large number of proteins. When viewed by SDS-PAGE, many of the observable bands contain multiple proteins, which can be digested simultaneously and identified using *laser induced dissociation* and MS/MS. Table 1 lists some of those identified from four of the bands observed in the *albuminome* from pig serum.

3. Conclusions

Mass spectrometry has definitively proven its worth in the area of *proteomics*. While one approach is to determine simple, disease-specific changes in protein expression, it is probably also necessary to provide identification, not only of the proteins themselves, but also of the many possible kinds of post-translational processing and modifications that may also be the significant biomarkers. From the mass spectroscopist's point of view, this requires many different kinds of measurements; and in this paper we have outlined some of the advantages of using laser-induced and high energy collision-induced dissociation on a tandem TOF, very high mass measurements with a cryocooled detector and reduction of the analytical measurement task itself utilizing “subproteomes” such as the *albuminome*.

QUESTIONS

Dr. Michael Gross: Your design of a time-of-flight appears to be one of the only instruments available today that does truly high-energy collisional activation. I think this is

important as this means of activation was formerly done with sector-field tandem instruments, and they are no longer commonly available. Do you agree?

Dr. Cotter: That is correct and the possibility for bringing high energy collisions to time-of-flight mass spectrometry was indeed our motivation for this work when we first proposed it for funding by NIH in 1992.

Dr. Gross: I would appreciate your opinion on the advantages of high-energy collisional activation on your design of a TOF (and on sectors) with respect to the lower energy activation done on triple quads, ion traps, and QTOFs.

Dr. Cotter: I have been most interested in the emergence of the new activation techniques, such as *electron capture dissociation* (ECD) that are supposed to remedy the problems of collision-induced dissociation. In particular, the assertion is that CID cannot determine posttranslational modifications (*e.g.*, phosphorylation, glycosylation, acetylation, etc.) because they are lost as the major fragmentation. This in fact is a property of low energy CID and other “thermal” methods such as *infrared multiphoton dissociation* (IRMPD) which activate ions through a series of collisions or interactions, and result in the cleavage of the weakest bond. Indeed most of this experience with which these methods are compared are with ion traps, QTOFs and FTMS in which activation involves repeated low energy collisions. Perhaps because there are very few high energy CID instruments available today, it is not appreciated that such collisions might indeed lead to the more widely distributed *charge-remote fragmentation* which you have described.

Dr. Gross: You spoke of the collisional activation of fullerenes. One signature of true high-energy collisional activation is the ability to insert into fullerenes a helium atom (when helium is the collision gas). Helmut Schwarz first published on this and we followed very closely with reports of the activation of C₆₀ and homologs. The sign for the insertion is that many of the peaks occur in pairs, separated by 4 u (the mass of helium). Those at higher *m/z* contain the He atom from the CAD process. Did you observe any insertion of He?”

Dr. Cotter: In our earlier instrument we did attempt this, but with no success. In part this was due to the very low mass resolution of that instrument. It would indeed be interesting to try that on the current tandem TOF.

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Fig. 1.

The 1993 tandem time-of-flight (TOF/TOF) mass spectrometer with two reflectron mass analyzers and a pulsed gas collision chamber. The second reflectron, which is used to focus product ions, is a curved-field reflectron.

Fig. 2.

An approach to the design of a tandem TOF mass spectrometer described in ref. ⁸, in which precursor ions are decelerated prior to entering the collision chamber, and the product ions are then extracted and reaccelerated from a pulsed “second source” before entering the reflectron.

Fig. 5.
Collision-induced dissociation, tandem mass spectra of buckminsterfullerene (C_{60}) at different pressures of helium collision gas.

Fig. 6. Attenuation of C₆₀⁺ precursor ions and formation of C₅₀⁺ and C₁₅⁺ ions as a function of helium gas pressure.

Fig. 7.
CID mass spectra of Substance P at different pressures of the helium collision gas.

Fig. 8. Laser-induced dissociation, tandem mass spectra of two peptides obtained from *histone acetyltransferase*. (a) HAT₂₋₁₇ and (b) HAT₁₄₉₋₁₆₄. (Reprinted with permission from ref. 18.)

Fig. 9.
LID tandem mass spectrum of HAT₂₃₈₋₂₅₆, which contains two proline residues. (Reprinted with permission from ref. ¹⁸.)

Fig. 10.

LID tandem mass spectrum of a tryptic peptide from histone *acetyltransferase* with two acetylated lysine sites. (Reprinted with permission from ref. ¹⁸.)

Fig. 11. LID tandem mass spectra of (a) the N-terminal sulfonated peptide LGIHEDSQNR and (b) the N-terminal sulfonated tryptic peptide from tetra-ubiquitin containing the ubiquitination site. (Reprinted with permission from refs. 19 and 20, respectively.)

Fig. 12.
Surface-enhanced laser desorption/ionization (SELDI) mass spectra of albumin-depleted and whole serum obtained on the Kratos AXIMA CFR.

Fig. 13.
Macromizer mass spectra of the “albuminome” and the albumin-depleted pellet.

Table 1Proteins Identified in the Albuminome^{a)}

Protein	Band	MW (kDa)	Fragments observed
Inter-alpha typsin inhibitor (H1) precursor	1	100.4	{ 787.0, 829.0, 866.9 , 931.2, 939.8, 1173.7, 1205.6, 1247.6, 1469.9 , 1473.1, 1629.8, 2037.6
Inter-alpha typsin inhibitor (H2) precursor	1	104.6	{ 821.0 , 891.9, 907.9, 975.8, 1337.6, 1452.4 , 1582.3, 1745.1 , 1803.9, 1807.9, 2239.4
Complement component C3	5	186.81	{ 787.2, 833.3 , 845.3, 860.3, 868.4, 887.3 , 900.4, 912.4 , 959.4, 1053.5, 1083.5 , 1099.6, 1126.5, 1143.5 , 1211.6, 1290.6 , 1457.8, 1669.8, 1683.9, 2170.4
Fibrinogen alpha chain—Fibrinopeptide A	8	1.76	{ 1091.4, 1761.6
Fibrinogen-A-alpha chain	8	47.33	{ 702.3, 848.1, 1100.5, 1107.4 , 1145.5, 1183.5, 1296.5, 1316.5, 1379.5, 1579.7
Thrombopoietin	8	2.9	{ 870.4
Apolipoprotein-E precursor	9	36.58	{ 886.4, 899.3 , 958.4, 968.4, 990.5, 1033.4 , 1077.4, 1146.5 , 1274.5, 1542.6 , 1743.6, 2288.8

^{a)} Corresponding band from 12% gel, molecular weight, and fragments observed are listed. Fragment masses for which sufficient information was obtained are in **bold**. Other masses were assigned to protein *via* reverse peptide mass fingerprinting.