Combined infrared multiphoton dissociation and electron capture dissociation with a hollow electron beam in Fourier transform ion cyclotron resonance mass spectrometry

Youri O. Tsybin1, Matthias Witt2, Gökhan Baykut2, Frank Kjeldsen1 and Per Håkansson1*

1Division of Ion Physics, The Ångström Laboratory, Uppsala University, Box 534, SE-751 21, Uppsala, Sweden
2Bruker Daltonik GmbH, Fahrenheitstrasse 4, D-28359 Bremen, Germany

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An electron injection system based on an indirectly heated ring-shaped dispenser cathode has been developed and installed in a 7 Tesla Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. This new hardware design allows high-rate electron capture dissociation (ECD) to be carried out by a hollow electron beam coaxial with the ion cyclotron resonance (ICR) trap. Infrared multiphoton dissociation (IRMPD) can also be performed with an on-axis IR-laser beam passing through a hole at the centre of the dispenser cathode. Electron and photon irradiation times of the order of 100 ms are required for efficient ECD and IRMPD, respectively. As ECD and IRMPD generate fragments of different types (mostly c, z and b, y, respectively), complementary structural information that improves the characterization of peptides and proteins by FTICR mass spectrometry can be obtained. The developed technique enables the consecutive or simultaneous use of the ECD and IRMPD methods within a single FTICR experimental sequence and on the same ensemble of trapped ions in multistage tandem (MS/MS/MS or MSn) mass spectrometry. Flexible changing between ECD and IRMPD should present advantages for the analysis of protein digests separated by liquid chromatography prior to FTICRMS. Furthermore, ion activation by either electron or laser irradiation prior to, as well as after, dissociation by IRMPD or ECD increases the efficiency of ion fragmentation, including the w-type fragment ion formation, and improves sequencing of peptides with multiple disulfide bridges. The developed instrumental configuration is essential for combined ECD and IRMPD on FTICR mass spectrometers with limited access into the ICR trap.

Peptide and protein characterization by Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) alone and combined with separation techniques is a powerful tool in proteomics research.1,2 Sequence verification, de novo sequencing, and characterization of post-translational modifications can be achieved by tandem mass spectrometry, based on collision-induced dissociation3 (CID), blackbody infrared dissociation4 (BIRD), infrared multiphoton dissociation5 (IRMPD) and electron capture dissociation6,7 (ECD). For experiments limited in time, IRMPD and ECD are the techniques of choice as no collision gas is required and analysis can be carried out on a millisecond time scale.

Structural analysis of peptides and proteins from complex biological mixtures can be performed using a combination of separation techniques, such as liquid chromatography8 (LC) or capillary electrophoresis9 (CE), together with tandem FTICRMS employing IRMPD10 or ECD.11–13 For tandem mass spectrometry of peptides separated by LC or CE and eluting during a time period of only 10–30 s, short fragmentation times are required. Current development of the ECD technique14 has shown that it is necessary to use indirectly heated dispenser cathodes for electron production in order to obtain reliable high-rate ECD.15

Conventionally, the implementation of IRMPD or ECD requires positioning of the IR-laser beam (Fig. 1(b)) or the low-energy pencil electron beam (Figs. 1(c) and (d)) on the axis of the ICR trap. Here we use the conventional notation of ‘pencil electron beam’ for the full cylindrical electron beam. When a directly heated filament is used for electron production in ECD experiments, it is possible to locate the filament off-axis and keep the laser beam on-axis.6,16 As directly heated filaments generate a very thin electron beam (Fig. 1(c)), the advantages of high collisional cross-sectional area and high electron flux produced by dispenser cathodes are lost. However, increasing the size of the dispenser cathode (Fig. 1(d)) eliminates the possibility of keeping both laser and electron beams close to the ICR trap axis. Numerous attempts to find the optimum position for the electron source
The ICR trap. This instrumental configuration provides radial position of the laser beam plus its entrance angle into attempted to keep the electron source on-axis and change the radial position of the laser beam plus its entrance angle into the ICR trap. Recent studies attempted to keep the electron source on-axis and change the radial position of the laser beam plus its entrance angle into the ICR trap. This instrumental configuration provides simultaneous access for both electron and photon beams to perform an on-axis high-rate ECD and off-axis IRMPD. Such a configuration is limited to FTICR instruments with a sufficiently large magnet bore diameter and preferably equipped with an open ICR trap.

This work presents an approach for simultaneous introduction of electron and photon beams into FTICR mass spectrometers with various magnet bore diameters and ICR traps with reduced entrance diameters (Fig. 1(a)). First of all, the performance of IRMPD with an on-axis IR-laser beam is demonstrated. Secondly, it is shown that high-rate ECD can be performed using an axially symmetric hollow electron beam produced by a ring-shaped dispenser cathode (Fig. 1(e)). Characteristic features and advantages for a system with simultaneous access for both electron and photon beam irradiation are discussed for analysis of peptides, proteins and peptide mixtures.

**EXPERIMENTAL**

**Samples and sample preparation**

Standard peptides and proteins, such as substance P (MW 1347), endotoxin inhibitor (MW 1245), hecate (MW 2537), defensin (MW 3442), ubiquitin (8.6 kDa), bovine carbonic anhydrase II (29 kDa) and human serum albumin (HSA, 66 kDa), were purchased from Sigma (St. Louis, MO, USA) and used without further purification. Tryptic decapetide from signal recognition particle (SRP) of *Saccharomyces cerevisae* (MW 1261) was synthesized in-house using an EPS221 automatic peptide synthesizer (Intavis AG, Cologne, Germany). The peptides and proteins were dissolved in a standard spraying solution (49:49:2 water/methanol/acetic acid, v/v/v) and diluted to final concentrations of 2 pmol/µL for substance P, SRP, endotoxin inhibitor, and 10 pmol/µL for hecate, ubiquitin and carbonic anhydrase. The HSA tryptic digest concentration was equivalent to 1 pmol/µL and the injected volume was 1 µL.

**Mass spectrometry**

All experiments were performed using an Apex III FTICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA) with a 7 Tesla superconducting magnet. Positive ions were produced in an external Apollo electrospray ion source (Bruker Daltonics) with a flow rate of 2 µL/min for direct infusion experiments and 200 nL/min for liquid chromatography (LC) experiments. The reversed-phase LC experiments were performed using the LC Packings Ultimate nano-LC system with Famos autosampler (LC Packings, San Francisco, CA, USA) according to methodology described elsewhere. 11 External accumulation of the electrosprayed ions in a hexapole ion trap prior to injection into the ICR trap was used in order to increase the sensitivity and duty cycle of the mass spectrometer. In direct infusion experiments an accumulation time of 1 s was used, unless otherwise stated. During liquid separation experiments the external accumulation time was reduced to 0.1 s by avoiding the hexapole quenching event.

Ions were captured in the ICR trap using the sidekick technique. This technique applies a voltage to ions entering the ICR trap that shifts them radially from the axis. Preliminary results showed the optimum value for the sidekick potential to be -6 V for both IRMPD and ECD experiments. This value was used throughout all experiments, unless otherwise stated.

In some experiments, ions of a desired charge state were isolated by the application of a pre-programmed waveform (correlated sweep technique) and the presence of a collision gas.

The efficiency of the dissociation process was determined by comparing the absolute total intensity of the fragment ions and the change in the absolute intensity of the precursor ions before and after the fragmentation.

**Photon irradiation**

Infrared multiphoton dissociation and activation of ions in the ICR trap were performed using a CO2 J48-2 laser with 25 W maximum power output (Synrad, Mukilteo, WA, USA). The laser was installed at the rear of the magnet. The laser beam was introduced into the ultrahigh vacuum system through a barium fluoride window and aligned along the ICR trap axis (Fig. 2). Photon irradiation time was varied from 10–300 ms.
Electron irradiation
A custom-designed indirectly heated ring dispenser cathode (HeatWave, Watsonville, CA, USA) with an i.d. of 3.5 mm and an o.d. of 7.6 mm was employed for electron production. Electrons are emitted from an indirectly heated tungsten surface impregnated with barium oxide to enhance electron emission. The cathode was installed behind the ICR trap at a distance of about 100 mm from the ICR rear trapping plate. During the ECD experiments the cathode heater current was set to 1.9 A corresponding to a voltage of 8 V and a cathode surface temperature of about 900°C. An external power supply was used to generate the current for the cathode heater. The electron-emitting surface potential was 18 V during ion injection and detection then pulsed to a computer-controlled potential during electron injection.

A cylindrical electrode (anode) was used to extract the electrons from the cathode surface. The anode was designed to have the same i.d. (6 mm) as the entrance hole of the ICR trapping plate in order to avoid exposure of the ICR trapping plate to electrons. The electrons were extracted by applying a potential difference of 8 V between the anode and the electron-emitting surface. The cathode-anode distance was 2 mm.

The electron current was measured on the anode and on the grounded collector temporarily placed on the ion source side of the ICR trap. The trapping plate, through which the ions enter the trap, was kept at 0.9 V and the opposite one, through which the electrons enter the trap, was kept at 1.1 V. The electron current after passing the ICR trap was about 100 mA for a cathode heater current of 1.9 A and cathode potential of −1 V. For the same experimental conditions, the electron emission current density from the cathode surface was about 10 μA/cm². As expected, the electron current in the hot ECD regime (electron energy is about 10 eV) is more than 100 times greater when compared to the conventional ECD regime (electron energy is below 1 eV).

RESULTS AND DISCUSSION
Hollow vs. pencil electron beam ECD
The design of the hollow electron beam injection system (Fig. 2) is based on the following requirements. First, it should permit on-axis transmission of an IR-laser beam, with a diameter of at least 3 mm, into the ICR trap. Secondly, the electron flux should be high enough and the overlap of ion and electron clouds in the ICR trap should be sufficient to produce efficient high-rate ECD. To compare the performances of ECD with the hollow electron beam (ring-shaped dispenser cathode) and ECD with an on-axis pencil electron beam (6 mm diameter full dispenser cathode), both electron injection systems were installed in similar 7 T FTICR mass spectrometers. At the ICR trap entrance, the cross-sectional area of a pencil electron beam is about 28 mm² and of the hollow electron beam is about 19 mm².

In both cases the ECD efficiency was optimized by tuning the electron energy by modifying the electron-emitting surface potential. The maximum efficiency was obtained with similar experimental parameters for both hollow and pencil electron beams. It was found that, for isolated and non-isolated precursor ions, ECD with either type of electron beam is efficient in a wide range of potentials (about 0.3 V wide at the 90% level). Successful ion fragmentation by electrons from such a wide range of emitting surface potentials can be explained by the relatively wide energy...
distributions of both electrons and ions in the ICR trap. This dependence also indicates that the electron energy dispersion is similar for both electron injection systems.

Furthermore, the results indicate that ECD with a hollow electron beam can be performed on the ions trapped without the sidekick and an additional RF-excitation of a cyclotron motion. However, the efficiency of ECD can be increased by using the sidekick trapping or ion excitation with an RF-voltage pulse. Using full and ring-shaped dispenser cathodes for ECD of standard peptides and proteins of equivalent amounts similar fragmentation patterns were obtained. Figure 3 also compares well with figures published in Refs. 15 and 18.

The ECD rate measurements for isolated substance P and non-isolated ubiquitin precursor ions were performed for both the hollow electron beam and pencil electron beam configurations. As expected, the rate of ECD with a hollow electron beam noticeably exceeds the rate of ECD reactions obtained by using a conventional heated filament for electron generation. With the optimum values for electron irradiation of 100 ms and electron-emitting surface potential of ~1 V the rate of ECD reaction obtained by using the ring cathode is comparable to the ECD reaction rate obtained by using the full cathode. In both cases, the electron irradiation time required for ECD of previously non-isolated ions is about half of that for isolated precursor ions due to the decreased ion-electron collision frequency after isolation procedure, which increases the energies and orbits of precursor ions.

**Independent IRMPD and ECD**

IRMPD spectra of isolated multiply charged ions of substance P, endotoxin inhibitor, SRP, hecate, ubiquitin and carbonic anhydrase were successfully obtained by irradiating the ions with an on-axis IR-laser beam. For 80% of the maximum laser power the optimum photon irradiation time was found to be about 90 ms for isolated precursor ions and even shorter for non-isolated precursor ions. With the same experimental parameters, ECD of similar precursor ions was achieved by a hollow low-energy electron beam without photon irradiation. The required electron irradiation times were similar to IR-laser irradiation times for both isolated and non-isolated precursor ions. The results clearly show the possibility of performing IRMPD (Fig. 4) and ECD (Fig. 5) of isolated ions in separate experiments for the same experimental parameters and without breaking the ultrahigh vacuum to change IRMPD and ECD hardware.

Ion irradiation with either an electron or a photon beam in one single experiment was used in the IRMPD/ECD experiments combined with LC/FTICRMS. For all LC/FTICRMS experiments a typical set of 400 mass spectra was acquired with 6–10 s accumulation time for each mass spectrum. Several experimental approaches were implemented for the analysis of HSA trypic digest separated by reversed-phase nano-LC. During some experiments the IRMPD or ECD processes were activated in alternating spectra. For other experiments these dissociation processes were separated by a mass spectrum without ion dissociation. Ion isolation was not applied during any of the LC/FTICRMS experiments. By using the appropriate cathode potential and power level of the IR-laser it was possible to keep both photon and electron irradiation times equal to 100 ms. The results indicate distinct advantages for alternating between ECD and IRMPD during the analyses of eluting peptides. Photon irradiation produces extensive peptide backbone fragmentation, including preferential cleavage of the N-terminal side of proline residues, allows fragmentation of singly charged ions and can indicate the presence of post-translational modifications. ECD, in turn, generates complementary sequence information, results in more efficient fragmentation of peptides with disulfide bridges, and also has potential for the characterization of post-translational modifications. ECD, in turn, generates complementary sequence information, results in more efficient fragmentation of peptides with disulfide bridges, and also has potential for the characterization of post-translational modifications. However, a further increase in tandem mass spectrometry sensitivity is required for application of the method for structural analysis of unknown biological samples.

The ability to perform both IRMPD and ECD on the same ensemble of trapped ions increases the application area of multistage tandem mass spectrometry (MS/MS, MS^n). This has previously been performed using the off-axis position of the conventional heated filament employed for electron
Figure 4. Tandem mass spectra of hecate showing (a) isolation of quadruply charged ions of hecate, (b) IRMPD of the isolated quadruply charged ions of hecate, and (c) its m/z 710–960 region. The photon irradiation time was 90 ms with a laser power of 80%. A total number of 128 single mass spectra were accumulated.
production (see Ref. 6 and Fig. 14 therein). The results of MS/MS/MS experiments presented in Fig. 6 reveal the sequence tag of \( c \)-fragments generated by ECD with a hollow electron beam (Fig. 6(b)) of the isolated doubly charged \( y_8 \) fragment of peptide SRP produced by IRMPD (Fig. 6(a)). The isotopic distributions of \( y_7^+ \) (Fig. 6(a)) and \( c_7^+ \) (Fig. 6(b)) ions demonstrate the performance of the technique in distinguishing between ions with close mass-to-charge ratios. The presence of \( a \) and \( b \) ions in the ECD spectrum (Fig. 6(b)) can be explained by collision-induced fragmentation of \( y_8^+ \) ions during gas-assisted ion isolation.

**Consecutive and simultaneous IRMPD and ECD**

Consecutive and simultaneous irradiation of trapped ions by both electrons and photons was performed after programming the appropriate sequence of experimental requests. The typical fragmentation mass spectrum obtained using consecutive photon and electron irradiation contains ions specific for both IRMPD and ECD fragmentation patterns (Fig. 7). This, very often complementary, sequence information can be used for unambiguous structural characterization of peptides and proteins. The fragmentation patterns obtained during simultaneous ion irradiation by both electrons and photons were similar to those obtained by using consecutive irradiation. A comparison of the fragmentation pattern shown in Fig. 7 with the MS/MS/MS results obtained by ECD of the isolated doubly charged IRMPD generated \( y_8 \) fragment (Fig. 6) indicates the presence of \( c \)-fragments from \( y_8^{2+} \) ions resulting from secondary fragmentation (assigned with an asterisk *) in the case of consecutive or simultaneous irradiation.

Ion activation by either electrons or photons prior to IRMPD or ECD reactions further extends the possibilities for peptide and protein fragmentation. Ion excitation by an RF-voltage pulse following the IR-photon irradiation increases the orbit of the ion packet causing further interaction with the electron beam21,25 (Fig. 1(e)). This leads to more efficient fragmentation of peptides and proteins, including the
Figure 6. MS/MS/MS mass spectrum obtained in an IRMPD/ECD experiment. The isolated $y_{8}^{2+}$ ions generated by IRMPD of peptide SRP (a) were fragmented by ECD (b) to produce $c$, $z$-fragments. The photon irradiation time was 100 ms at 80% power level. The electron irradiation time was 100 ms and the electron-emitting surface potential was –1 V. 128 single mass spectra were accumulated. Fragment ions assigned with an asterisk (*) are the result of a secondary fragmentation of $y_{8}^{2+}$ ions.

Figure 7. Tandem mass spectrum of isolated doubly charged ions of the peptide SRP obtained by consecutive IR-photon and low-energy electron irradiation. Intensities of $w_{4}$-ions were monitored as a function of IR-laser irradiation time prior to ECD. The insert shows the region of the mass spectrum around the $w_{4}$-ion position in (a) the IRMPD regime and (b) the ECD regime. A total number of 32 single scans was accumulated. Fragment ions assigned with an asterisk (*) are the result of a secondary fragmentation of $y_{8}^{2+}$ ions.
analysis of peptides with three or more disulfide bridges and characterization of post-translational modifications. Results were obtained supporting the previously reported increase in the intensities of fragments of both c, z (ECD) and b, y (IRMPD) ion types due to the consecutive irradiation of ions with electrons and photons. Here we report on the substantial increase in intensity (80%) of secondary fragments (e.g. w₄-ions in Fig. 7) by simultaneous or consecutive irradiation with photons and electrons of isolated doubly charged ions of peptide SRP. For the particular sample studied, no w₄-ions are observed when only IRMPD is applied (Fig. 7, insert (a)). In the ECD spectra of previously isolated doubly charged SRP ions (Fig. 7, insert (b)), w₄-ions of low intensity are observed. The most efficient w₄-ion formation was obtained when 140–160 ms IR-laser irradiation at 50% power was used for ion activation prior to ECD. A more detailed investigation into w-ion formation by combined action of electron and photon beams is currently underway. The presence of w-ions in the tandem mass spectra not only provides complementary sequence information, but also allows for distinguishing between isomeric Ile and Leu residues.

Combined electron and photon irradiation for sequencing of the peptide defensin (human neutrophil peptide-1) is demonstrated in Figs. 8 and 9. Several peptide fragmentation techniques have been applied for sequencing of defensin, including CID, multipole storage assisted dissociation (MSAD) and IRMPD (Fig. 8(a)). However, the tightly folded structure of defensin, containing three disulfide bridges, did not allow fragmentation of the peptide or to efficient neutralization of the charge state. The application of electron irradiation alone led to efficient charge state neutralization with production of few c- and z-fragments. Furthermore, the combined action of electrons and photons generated more reliable fragmentation spectra (Figs. 8(b) and 9) with more than 60% sequence coverage. Due to the fact that no ion
isolation was performed prior to tandem mass spectrometry of defensin, the modified defensin (M', 150 Da heavier than M) is also present in mass spectra (see Fig. 8).

The application of the ‘top down’ tandem mass spectrometry approach, developed by McLafferty and co-workers, using combined electron and photon irradiation for the analysis of larger proteins, including bovine carbonic anhydrase II (Fig. 10), have produced results similar to those obtained using activated ion ECD (Ref. 28 and Fig. 1 therein). The higher electron flux generated by the indirectly heated
dispenser cathode allowed ECD to be performed without introducing collisional gas for ion activation.

CONCLUSIONS

A novel instrumental configuration designed to combine electron (ECD) and IR-laser (IRMPD) irradiation of ions in an ICR trap has been implemented and applied to structural analysis of peptides and proteins infused into the FTICR mass spectrometer directly and after separation by LC. The IRMPD was performed using the conventional experimental configuration with an on-axis IR-laser beam. High-rate ECD was achieved by employing a ring-shaped dispenser cathode to produce a hollow electron beam coaxial with the ICR trap. This new experimental setup is essential for combining IRMPD and ECD on instruments with small magnet bore diameters and ICR traps with limited access for laser or electron beams.

The consecutive or simultaneous use of ECD and IRMPD is possible within a single FTICR experimental sequence, hence on the same ensemble of the trapped ions in MSn experiments. Ion activation/dissociation by either electrons or photons prior to IRMPD or ECD reactions further extends the capabilities of this technique for peptide and protein fragmentations. Improved structural analysis of peptides and proteins infused into the FTICR mass spectrometer directly and after separation by LC. The consecutive or simultaneous use of ECD and IRMPD is demonstrated.

The increased ECD rate employed in experiments with consecutive or simultaneous irradiation will support the fragmentation studies of heavy ions, be employed for protein folding/unfolding dynamics analysis, and characterization of post-translational modifications in top-down mass spectrometry.

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