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IRMPD and ECD fragmentation of intermolecular cross-linked peptides

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Despite the increasing number of studies using mass spectrometry for three dimensional analyses of proteins (MS3D), the identification of cross-linked peptides remains a bottleneck of the method. One of the main reasons for this is the lack of knowledge about the fragmentation of these species. Intermolecular cross-linked peptides are considered the most informative species present in MS3D experiment, since different peptides are connected by a cross-linker, the peptides chain can be either from a single protein, providing information about protein folding, or from two different proteins in a complex, providing information about binding partners, complex topology and interaction sites. These species tend to be large and highly charged in ESI, making comprehensive fragmentation by CID MS/MS problematic. On the other hand, these highly charged peptides are very suitable for dissociation using both infrared multiphoton dissociation (IRMPD) and electron capture dissociation (ECD). Herein, we report the fragmentation study of intermolecular cross-linked peptides using IRMPD and ECD. Using synthetic peptides and different commercial cross-linkers, a series of intermolecular cross-linked peptides were generate, and subsequently fragmented by IRMPD and ECD in a FT-ICR-MS instrument. Due to the high mass accuracy and resolution of the FT-ICR, the fragment ions could be attributed with high confidence. The peptides sequence coverage and fragmentation features obtained from IRMPD and ECD were compared for all charge states.

Supporting information may be found in the online version of this article.

Keywords: intermolecular cross-linked peptides; cross-linking; fragmentation; IRMPD; ECD

Introduction

Mass spectrometry for three-dimensional analysis of proteins (MS3D)[1] has been used in the study of three-dimensional structures of proteins. In these experiments, a chemical cross-linker is added to the solution containing the protein (or protein complex) of interest, followed by enzymatic digestion and MS analysis. Several different information can be obtained from this approach: study of protein folding,[1–6] identification of binding partners,[7–9] monitoring of conformational changes upon ligand binding,[10–13] characterization of surfaces in protein complexes[14–20] and probing for solvent residues accessibility.[21–24]

Despite the increasing number of studies using cross-linking coupled to MS,[2,5,9,11,12,19,25–28] the interpretation of cross-linked peptides spectra is not trivial. The most used cross-linkers posses a *N*-hydroxysuccinimide (NHS) moiety which will react preferentially with free amino groups (N-terminus and Lys residues) and a new amide bond is formed between the peptide residues and the cross-linker. This allows fragmentation pathways different from linear peptides and there are some works that report how the cross-linker presence influences fragmentation (most of them uses collision-induced dissociation, CID).[29–33] In the case where two different peptides are connected by a cross-linker (intermolecular cross-linked peptides or type 2 peptides[29]), peptide chains can be either from a single protein, providing information about protein folding, or from two different proteins in a complex, providing information about binding partners and complex topology. Intermolecular cross-linked peptides tend to be relatively large and highly charged in ESI, making comprehensive fragmentation by CID MS/MS problematic.[34]

The ability to extensively fragment such large, highly charged peptides can, in principle, be achieved by infrared multiphoton dissociation (IRMPD)[35] and/or electron capture dissociation (ECD),[36,37] whereas the high resolution and mass accuracy of Fourier transform-mass spectrometry (FT-MS) make it a powerful tool for unambiguous assignment of cross-linked peptides. As ECD fragments almost exclusively at the peptide backbone,[38,39] it has been extensively used for localization of post-translational modification (PTM)[40–42] but, to the best of our knowledge, there are few studies reporting the use of ECD or IRMPD for studies of cross-linked peptides.[34,43]

In the present work, we report the fragmentation study of intermolecular cross-linked peptides using IRMPD and ECD. Using synthetic peptides and three different commercial cross-linkers, a series of intermolecular cross-linked peptides were generated, different charge states were selected and subsequently fragmented by IRMPD and ECD in a FT-ICR-MS instrument. Due to the high mass accuracy and resolution of the FT-ICR, the fragment ions could be attributed with high confidence. The peptide sequence coverage obtained from IRMPD and ECD was compared for all charge states observed from each peptide.

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Experimental

Materials

The N-terminus acetylated peptides used here, ARAKGAEFAVYAGVR (P1, $[M + H]^+$ 1607.8658), ARAKGAEFAVHAGVR (P2, $[M + H]^+$ 1581.8614), ARAKGAEFAVSAGVR (P3, $[M + H]^+$ 1531.8345), ARAYVALKA (P4, $[M + H]^+$ 1004.5893) and ARAHVALKA (P5, $[M + H]^+$ 978.5849) were obtained from Proteimax (Campinas, Brazil). The cross-linkers disuccinimidyl suberate and disuccinimidyl sebacate (DSS and DSSeb, respectively) were obtained from Sigma–Aldrich (St, Louis, MO, USA) and disuccinimidyl glutarate (DSG) from Pierce (Rockford, IL, USA). All other reagents were obtained from Tedia Chemicals (Cincinnati, OH, USA) and used without further purification.

Cross-linking reactions

The intermolecular cross-linked peptides were formed between the following peptides P1 and P2; P3 and P4; P3 and P5; P1 and P3; P2 and P3. Reactions were performed by allowing 200 nmol of total peptide (100 nmol from each peptide) to react with 200 nmol of cross-linker (DSS, DSSeb and DSG). The reactions were performed in 50 µl of phosphate buffer (pH 7.5). The cross-linkers were added to generate the 1 : 1 cross-linker/peptide pair ratio from a cross-linker stock solution of 20 mg/ml in DMF and the reactions occurred at room temperature for 1 h. After the first hour, the same amount of cross-linker was added to each solution and the reaction vessels were kept at room temperature for another 1 h. After that, tris buffer (1 M, pH 7.6) was added to a final concentration of 50 mM. Free N-terminus intermolecular cross-linked peptides were generated from the reactions between P1 and P2; P1 and P3; P2 and P3 with DSS and enzymatically digested as follows: trypsin was added to a final concentration of 1 : 50 (weight) and the reaction was incubated at 37 °C for 3 h.

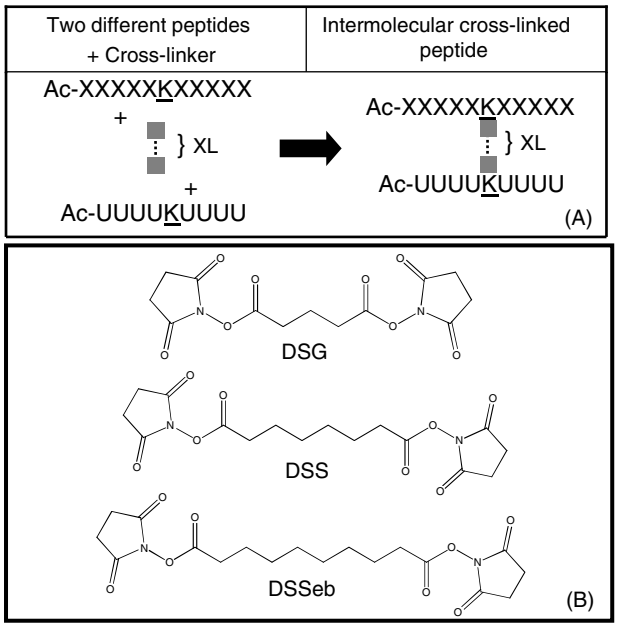
Sample preparation and MS analysis

Before MS analysis, all reaction products were desalted using an Oasis HLB Cartridge (Waters, Milford, MA, USA) made of a polymeric resin, endcapped with N-vinylpyrrolidone and divinylbenzene, according to the manufacturer's protocol. Samples were dried in a vacuum centrifuge and resuspended in 50% ACN/0.1% trifluoroacetic acid. The nano-ESI-MS/MS analysis was performed in a 7 T LTQ FT Ultra (Thermo Scientific Co., San Jose, CA, USA). Nanoelectrospray was performed on a NanoMate TriVersa (Advion Biosciences, Ithaca, NY, USA), with a capillary voltage of 1.5 kV. Before cross-linked peptide analysis, the instrument was calibrated using a mixture of caffeine, MRFA peptide and Ultramark 1621 (all from Sigma–Aldrich). The IRMPD MS/MS experiments were performed maintaining the laser energy at 100 and varying the exposure to laser from 20 to 200 ms and ECD MS/MS data were acquired fixing the electron energy at 4 and varying the exposure to electrons from 20 to 200. All spectra were manually interpreted.

Results and Discussion

Peptides, cross-linkers and nomenclature

All synthetic peptides used here have acetylated N-terminus, consequently, the ε-NH2-group from lysine residue should be the preferential site to react with the NHS esters, using the experimental conditions described above.[44] The protocol used to obtain the intermolecular species and the cross-linkers structures used here (DSG, DSS and DSSeb) are shown in Scheme 1(A) and (B), respectively. To identify the intermolecular cross-linked species and the respective fragments studied here, the nomenclature proposed by Madler *et al*.[45] was used and the MS/MS data were annotated according to Schilling *et al*.[29] The use of similar cross-linkers, which differ from each other by the space arm length, worked, therefore, as a mass shift reagent and it helped in the MS/MS interpretation from each peptide pair: fragments that contain the cross-linker should be modified by a delta mass corresponding to the difference of cross-linker masses. For example, fragments containing the cross-linker moiety from cross-linked peptide P1-DSS-P2 should have a mass +42 Da higher than the corresponding fragment from P1-DSG-P2, and so on.



Scheme 1. Formation of intermolecular species (A) and cross-linkers structures (B).

Using the protocol shown in Scheme 1(A), the following intermolecular species were studied: P1-DSG-P2; P1-DSS-P2; P1-DSSeb-P2; P3-DSG-P4; P3-DSS-P4; P3-DSSeb-P4; P3-DSG-P5; P3-DSS-P5; P3-DSSeb-P5; P1-DSS-P3 and P2-DSS-P3. Briefly, three similar intermolecular species (P1-XL-P2; P3-XL-P4 and P3-XL-P5) containing the different cross-linkers were generated (DSG, DSS and DSSeb). Moreover, P1-DSS-P2, P1-DSS-P2 and P2-DSS-P3 species were then digested using trypsin, and new, free N-terminal species were obtained. The IRMPD and ECD of the free and blocked N-terminal were then compared.

IRMPD of intermolecular cross-linked peptide

The highest charge state for each cross-linked peptides was fragmented by IRMPD. Figure 1(A–C) shows the $[M + 5H]^{5+}$ IRMPD spectra from P1-XL-P2 with the different cross-linkers: DSG (*m/z* 657.75), DSS (*m/z* 666.16) and DSSeb (*m/z* 671.77) from *m/z* 680 to 1150, the most populated fragment ion region. The fragmentation of P1-XL-P2 cross-linked peptide showed the formation of regular *b* and *y* ions for both α and β chains. An important feature observed here is that fragmentation occurs exclusively in one of the chains, whereas the other remains intact. This behavior was observed for both chains and it is exemplified in fragment

ions $b_{6\alpha}$ and $y_{13\alpha}$, which contain the cross-linker moiety and the intact β chain. Similarly, fragment ions $b_{4\beta}$ and $y_{13\beta}$ contain the cross-linker moiety and the intact α chain. The lack of parallel fragmentation pathway causes these fragment ions to be large and highly charged. This is in contrast to previous studies using CID, where parallel fragmentation was observed for intermolecular cross-linked peptides, especially when both chains have similar sizes.[33,44]

Figure 2(A) illustrates the sequence coverage obtained for the $[M + 5H]^{5+}$ P1-XL-P2 with DSG (dashed traces), DSS (black traces) and DSSeb (gray traces). The difference in cross-linker sizes did not affect the fragmentation behavior, and all cross-linked peptides showed very similar fragmentation spectra. Another fragmentation feature observed for IRMPD of intermolecular cross-linked peptides is the presence of intact chains ions: *m/z* 804.43 (2+) for the α chain and *m/z* 791.43 (2+) for the β chain (it stands out in the spectrum of P1-DSS-P2, Fig. 1(B) and it is less intense for P1-DSG-P2 and P1-DSSeb-P2, Fig. 1(A) and (C), respectively).

To evaluate the influence of N-terminal blocking, the $[M + 4H]^{4+}$ precursors of tryptically digested, free N-terminus P1-DSS-P2, P1-DSS-P3 and P2-DSS-P3 were compared to N-terminal blocked analogous. The fragmentation pattern was not altered by N-terminal blocking, causing both species to have same sequence coverage (Supporting Information Fig. 1).

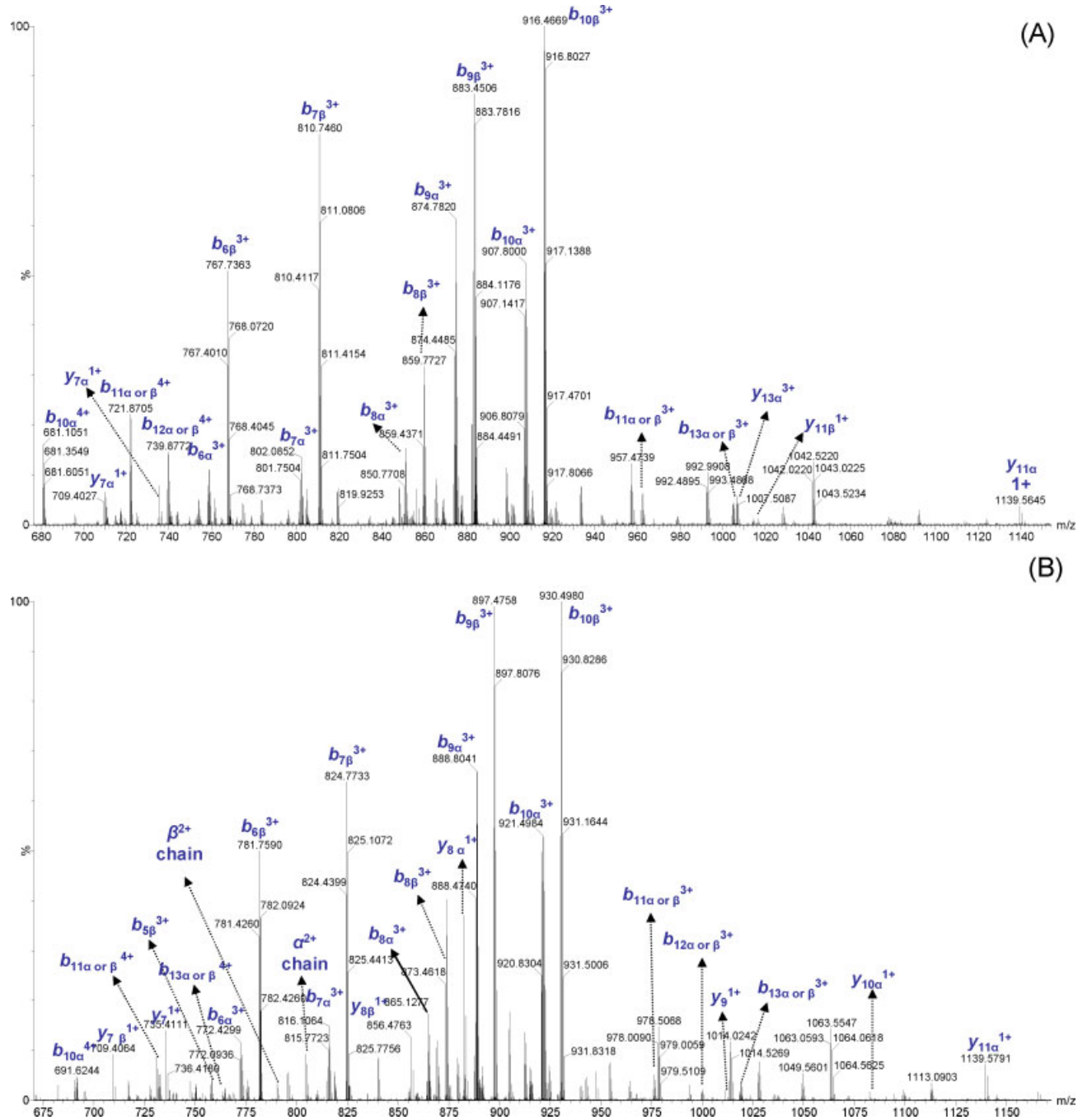


Figure 1. $[M + 5H]^{5+}$ P1-XL-P2 IRMPD attributed spectra using the cross-linkers DSG (A), DSS (B) and DSSeb (C).

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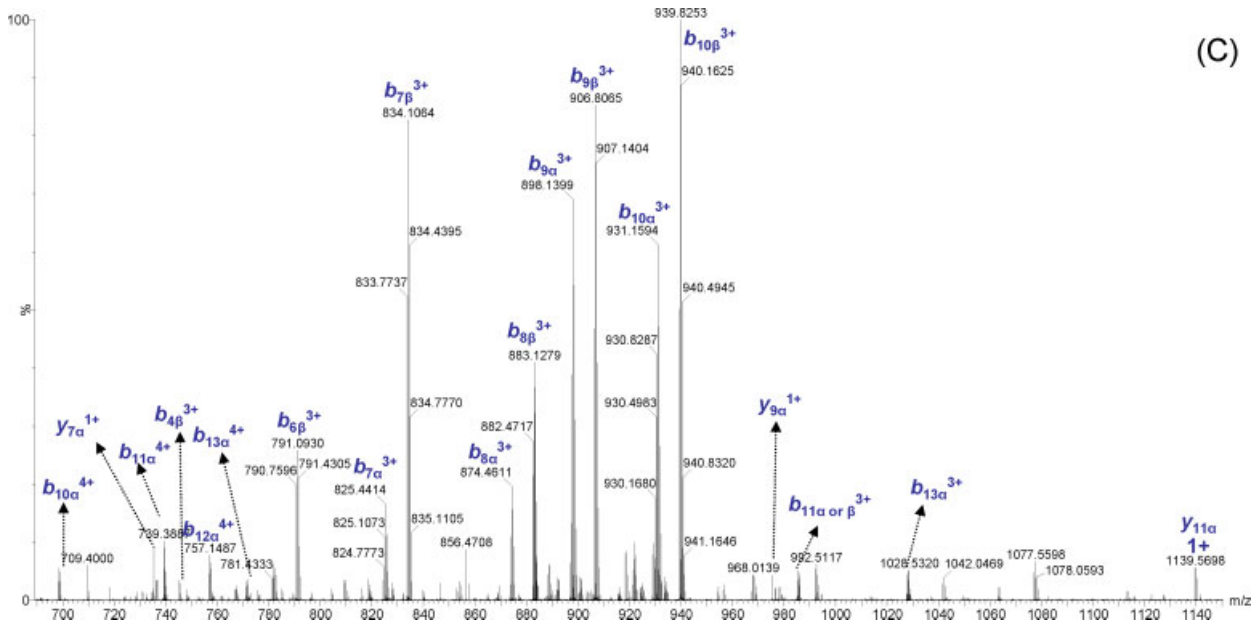

Figure 1. (*Continued*).

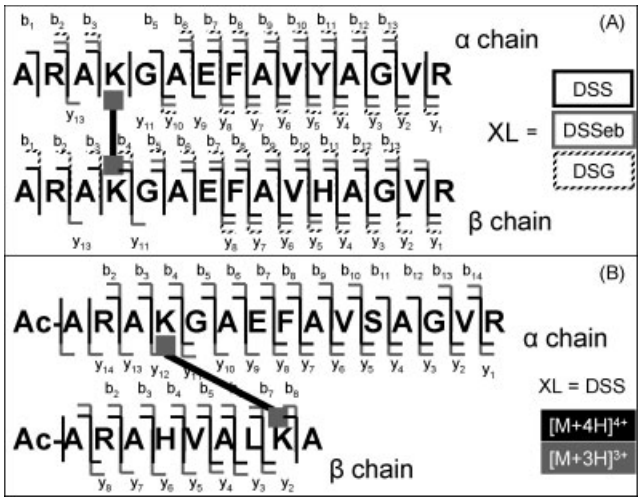

Figure 2. Sequence coverage obtained for: (A) $[M + 5H]^{5+}$ P1-XL-P2 IRMPD using the cross-linkers DSG (dashed traces), DSS (black traces) and DSSeb (gray traces); (B) P3-DSS-P5 IRMPD quadruply (black traces) and triply charged (gray traces).

Influence of charge states was also evaluated. Figure 2(B) shows the fragmentation coverage obtained for P3-DSS-P5 with three (*m/z* 883.16, gray traces) and four charges (*m/z* 662.63, black traces) and, except for the ions $y_{14\alpha}$, $y_{5\beta}$, $y_{6\beta}$ and $y_{7\beta}$ from the $[M + 3H]^{3+}$ and $b_{11\alpha}$ and $b_{12\alpha}$ from the $[M + 4H]^{4+}$, all other ions were present in both IRMPD spectra. Fragments with two charges were dominant in the $[M + 3H]^{3+}$ IRMPD spectrum while in the $[M + 4H]^{4+}$ spectrum, fragments with two and three charges were equally present (Supporting Information Fig. 2). The same comparison was done to the different charge state of all intermolecular cross-linked peptides, and no significance difference was observed between the sequence coverage obtained for the charge states 3+ and 4+ (and 5+ for the species from P1-XL-P2).

ECD of intermolecular cross-linked peptides

The highest charge state of each cross-linked peptide was selected for ECD experiments. As a general pattern, ECD presented low dissociation efficiency, causing the fragment ions to have low intensity when compared with the precursor ion. This trend has also been observed for ECD of intact proteins.[36,37,46] Excluding the precursor ion, the most intense ion corresponded to the electron attachment to the precursor ion forming the $[M + nH]^{(n-1)+\cdot}$, whereas the ion resulting from the attachment of two electrons to the precursor ion ($[M + nH]^{(n-2)+\cdot\cdot}$) was observed in a lower intensity. Figure 3 shows the ECD spectrum of P1-DSS-P2 in three different *m/z* ranges: from *m/z* 150 to 660 (A), 670 to 790 (B) and 1000 to 1610 (C). Interestingly, fragment ions corresponding to intact α (*m/z* 1607.9, 1+) and β chains (*m/z* 1581.9, 1+ and *m/z* 791.5, 2+) were present as fragment ions (other ECD data are present in Supporting Information Fig. 3).

As illustrated in Fig. 4(A), the coverage obtained by ECD of P1-XL-P2 (DSS, black traces; DSSeb gray traces and DSG, dashed traces) was close to 100% (*c* and *z* ions) and the cross-linker did not affect the fragmentation coverage. As in IRPMD, this high sequence coverage was also not altered by the blockage of N-terminus (Supporting Information Fig. 4).

The charge state, on the other hand, showed to have a large influence on the fragmentation behavior of cross-linked peptides. This is illustrated for P3-DSSeb-P5 with three (*m/z* 892.5) and four (*m/z* 669.6) charges. Quadruply charged P3-DSS-P5 presented doubly and triply charged fragments, whereas the triply charged precursor ion generated mostly singly charged fragments (Supporting Information Fig. 5). A fragmentation coverage close to 100% was observed for $[M + 4H]^{4+}$, whereas $[M + 3H]^{3+}$ showed lower coverage, especially for the ion series from $z_{3\beta}$ to $z_{6\beta}$ and from $c_{8\alpha}$ to $c_{14\alpha}$ (Fig. 4(B)).

General fragmentation trends

Both IRMPD and ECD showed a great fragmentation coverage improvement over CID, especially for the most highly charged

species. CID is known to present a lower fragmentation coverage as the peptide becomes larger and more highly charged, so both methods are attractive alternatives for the study of cross-linked peptides. The fragmentation features exemplified here were present in all other cross-linked peptides studied in the present work, so they seem to be a general trend for large, highly charged cross-linked peptides.

IRMPD showed to be a more robust dissociation technique regarding structural changes in the cross-linked peptide. The fragmentation coverage was mostly unaffected by N-terminal blocking, precursor charge state or cross-linker size. ECD also presents a very similar fragmentation pattern for both cross-linked peptides presenting free or acetylated N-terminus or different cross-linkers, but the fragmentation coverage was significantly increased for higher charge states. In fact, almost complete fragmentation coverage was obtained for 4+ and 5+ precursors, making this dissociation technique very attractive to study cross-linked peptides, since they are normally observed as highly charges species.

Unlike CID, parallel fragmentation[29,44] was not observed for both IRMPD and ECD, making the fragmentation spectrum less difficult to interpret. Ions corresponding to intact α and β chains were present in both dissociation techniques and they are useful fragments for structural diagnostics.

Conclusions

This work reports a fragmentation study of intermolecular cross-linked peptides by IRMPD and ECD methods, using model peptides

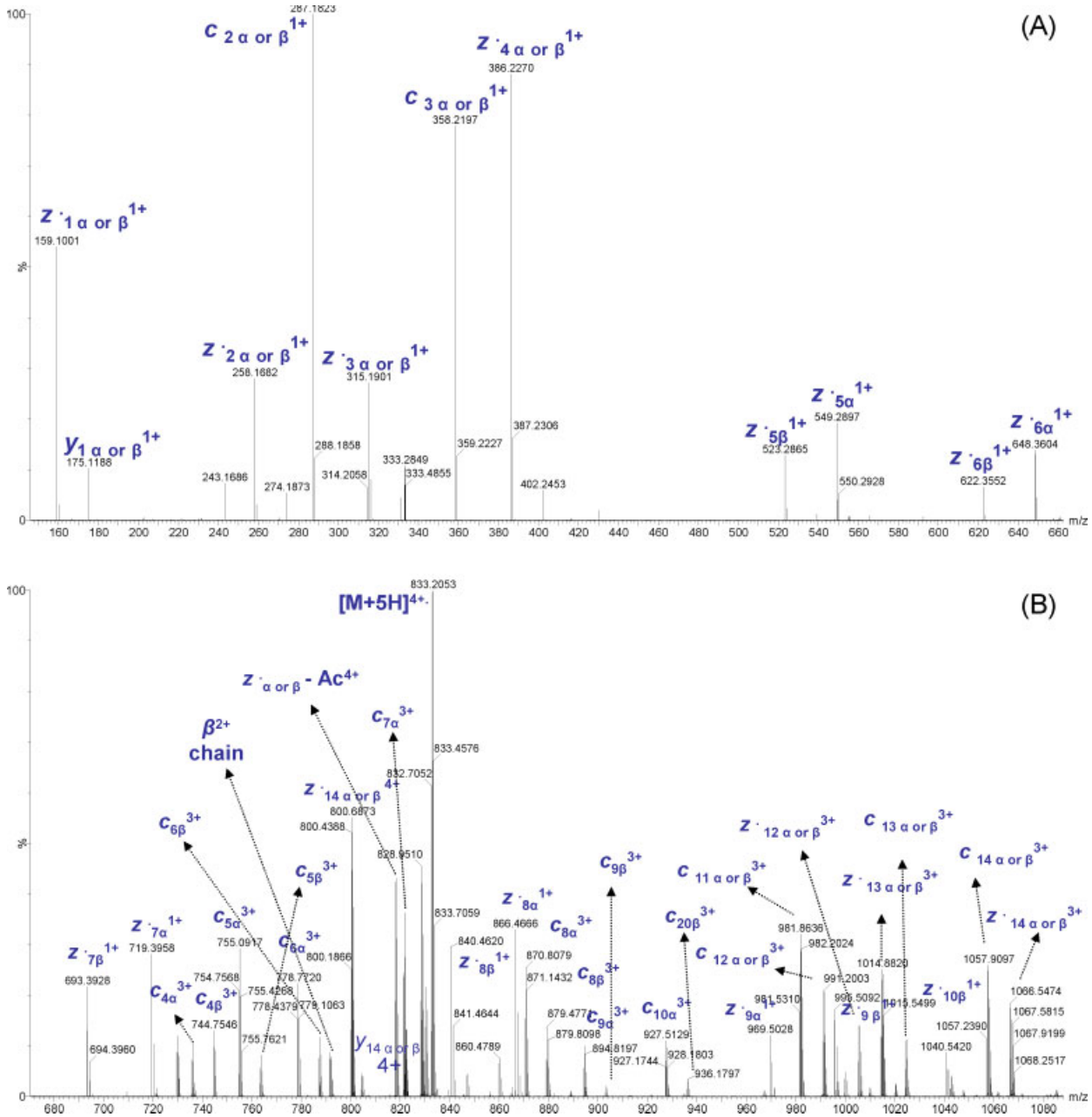

Figure 3. Expanded regions of $[M + 5H]^{5+}$ P1-DSS-P2 ECD spectrum from *m/z* 150 to 660 (A), *m/z* 670 to 790 (B) and *m/z* 1000 to 1610 (C).

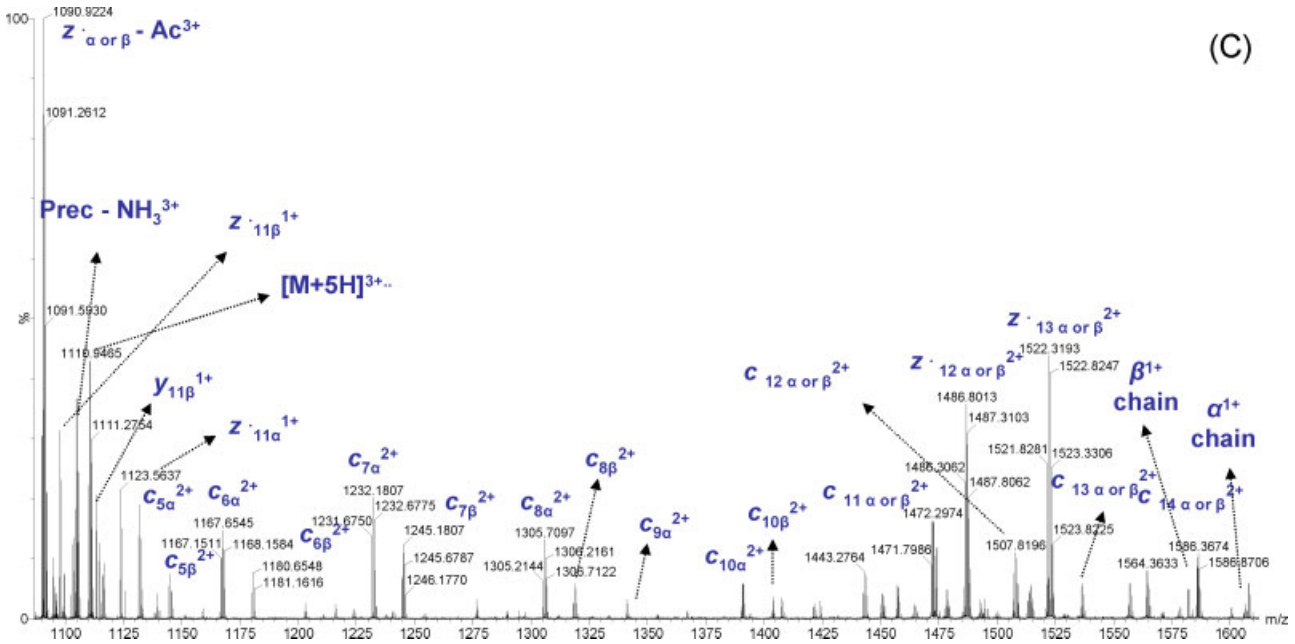

Figure 3. (*Continued*).

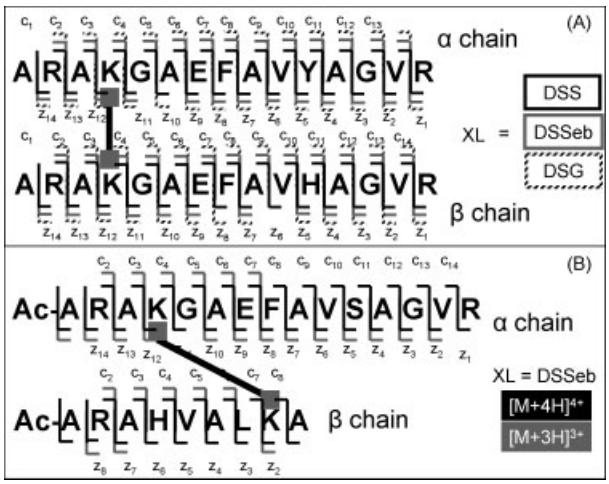

Figure 4. Sequence coverage obtained for: (A) $[M + 5H]^{5+}$ P1-XL-P2 ECD using the cross-linkers DSG (dashed traces), DSS (black traces) and DSSeb (gray traces); (B) P3-DSSeb-P5 ECD quadruply (black traces) and triply charged (gray traces).

and three different cross-linkers. Both methods showed to be attractive alternatives to CID in cross-linked peptides studies, since such species are usually large and highly charged, two features that are known to contribute to a poor fragmentation spectrum when using CID. Both methods are robust regarding structural changes in peptides chains (e.g. cross-linker size, N-terminal blocking, peptide sequence), making them applicable to a wide range of cross-linked peptides.

Fragmentation by ECD showed the highest fragmentation coverage, which increases with the charge state. This is a very useful dissociation technique, as the cross-linked peptides are usually highly charged, making the technique very suitable for structural proteomics studies using cross-linking.

Acknowledgements

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Supporting information

Supporting information may be found in the online version of this article.

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