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COMMUNICATION

Increased Disulfide Peptide Sequence Coverage via “Cleavage ON/OFF” Switch during Nano-electrospray

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Herein, we developed an in-situ disulfide cleavage protocol. It is based on the selective reaction between disulfide and the hydroxyl radical, which could be rapidly and controllably generated via induced nano-electrospray. In less than one minute, increased disulfide peptide sequence coverage could be obtained via switching the “cleavage ON/OFF” modes between conventional and induced nano-electrospray.

Identification of posttranslational modifications¹⁻⁴ is of increasing importance in functional proteomics studies, since posttranslational modifications reversibly or irreversibly alter the nature of proteins by chemical modifications. Disulfide bond (DSB) formation, one of the most known posttranslational modifications, is crucial for stabilizing tertiary structures of proteins⁵⁻⁹ and modulating activity of enzymes.¹⁰ As a promising tool for DSB-linked protein identification, mass spectrometry (MS) benefits widely from the striking capability of mass- and structure-accessibility. However, DSB would “bury” backbone sequence information in typical collision induced dissociation-mass spectrometry (CID-MS) since CID does not produce informative fragments if DSB remains intact. Thus, cleavage of DSB is normally mandatory prior to CID-MS identification since it would release “buried” peptide backbone.¹¹ In situ DSB cleavage method should enable MS to obtain the actual implementation in the realm of top-down^{2, 12-14} or bottom-up¹⁵⁻¹⁷ proteomics, taking de novo sequencing as an example.

The majority of classic DSB cleavage methods include chemical reduction using Tris (2-carboxyethyl) phosphine¹⁸⁻²⁰ or dithiothreitol (DTT)²¹ and alternatively electrochemical reduction.²²⁻²⁵ To avoid time- and sample-consuming wet chemistry, MS-based DSB cleavages have been increasingly reported, such as electron capture dissociation (ECD),^{26, 27} electron transfer dissociation (ETD),^{28, 29} in-source decay,³⁰ reduction with reducing matrix in MALDI³¹ and thermal dissociation.³² Previously, ultraviolet (UV) light photodissociation of DSB in gaseous proteins was reported,³³ in which efficient DSB cleavage was performed as well as some backbone cleavages, and instrumentation modification is necessary to guild UV light into ion trap mass spectrometer. Interestingly, oxidative cleavage based on selective oxidation between hydroxyl radical (HO•) and DSB was reported by Xia et al. for selective DSB cleavage free of backbone damage.^{34, 35} The cleavage products,

radical additive ions,³⁴ reveal backbone sequence using low temperature plasma as additional HO• source during conventional nano-electrospray ionization (nanoESI). Recently, reactive oxygen species (such as HO•) was also reported to be produced during conventional ESI via corona discharge.³⁶ Herein, we aimed at using induced nano-electrospray ionization (InESI)^{37, 38} source to provide HO• for instant and selective cleavage of DSB in peptides/proteins without extra plasma equipment.

In present work, this goal has been accomplished via in situ HO• generation by enhanced corona discharge³⁹⁻⁴⁵ during the InESI process (Fig. 1a). HO• could be controllably generated through regulating the amplitude and frequency of pulsed high voltage.

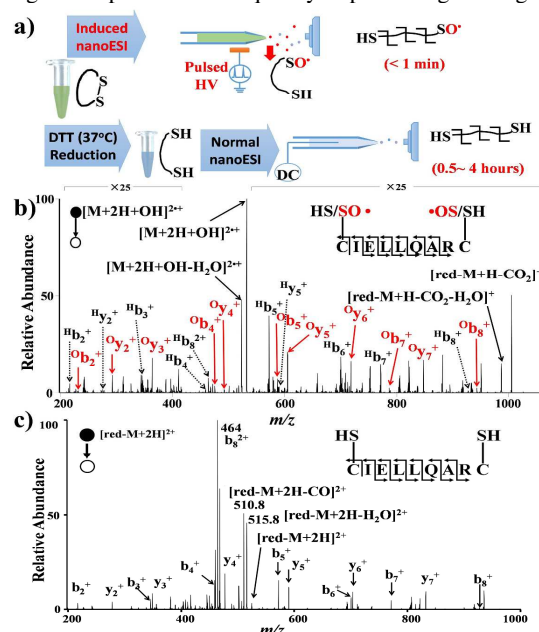


Fig. 1 Experimental scheme and CID spectra of selectin binding peptide. a) Comparison of InESI-MS (above) and classic DTT reduction nanoESI-MS (below) in total time required. CID spectra of selectin binding peptide via b) radical additive ion derived from InESI (pulse, 1 kHz, 6 kV) and c) protonated ion from DTT reduction nanoESI (DC, 1.5 kV).

Selectin binding peptide (${}_S\text{CIELLQARC}_S$), whose sequence is fully protected by DSB, was tested by InESI-CID-MS protocol. Water solution, containing 10 mM ammonium acetate (NH_4OAc), was used as the spray solvent, and it required less than one minute for whole InESI-CID-MS experiment. Upon turning on the pulsed voltage, sulfanyl radicals were observed under typical operation condition (${}_{\text{HS}}\text{CIELLQARC}_{\text{SO}}/{}_{\text{OS}}\text{CIELLQARC}_{\text{SH}}$) (the above path in Fig. 1a), as evidenced by the MS spectrum (Fig. S1) and corresponding tandem MS spectrum of the radical ion ($[\text{M} + 2\text{H} + \text{OH}]^{2+}$) (Fig. 1b). Sequence coverage of selectin binding peptide from the present method was similar to that from conventional nanoESI-MS method (Fig. 1c) following chemical reduction via DTT (the below path in Fig. 1a), which would be tedious (cost 0.5–4 hours) and inconvenient.²² Homolytic cleavage of the DSB was identified with the MS³ spectra of the oxygen addition fragment ions (Oy_2^+ , Oy_3^+ , Oy_4^+ , Oy_5^+ , Oy_6^+ and Oy_7^+). As shown in Fig. S2, the 62 Da neutral loss, which is attributed to the loss of CH_2SO from the oxidized cysteine residue, was observed as the major fragmentation pathway. Apart from selectin binding peptide, another peptide with an intra-disulfide chain (somatostatin, $\text{AG}_5\text{CKNFFWKTFTSC}_5$) (Table S1) was observed to undergo similar DSB cleavage pathway and increased sequence coverage was also achieved with the present method.

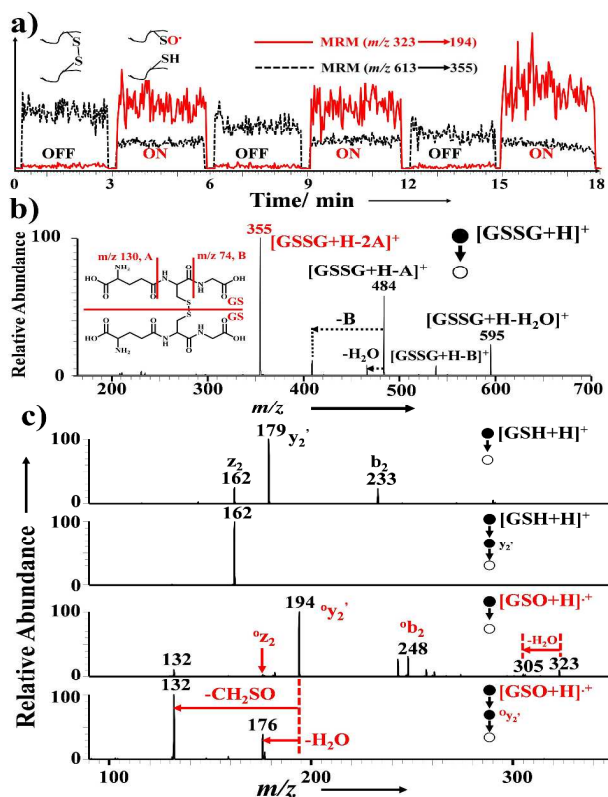


Fig. 2 “Cleavage ON/OFF” experiment with a) switch between InESI and nanoESI for GSSG analysis. b) GSSG (m/z 613, CID m/z 355) and c) $\text{GSO}\cdot$ (m/z 323, CID m/z 194) were monitored in multiple reaction monitor mode. For “cleavage ON” mode was operated as follows, pulsed high voltage (1 kHz, 7 kV) was applied on the ring electrode outside the tip for InESI operation and DC high voltage for conventional nanoESI was turned off. For “cleavage OFF” mode, DC high voltage (1 kV) was applied on the electrode inside the tip for nanoESI operation and pulsed high voltage for InESI was turned off. NH_4OAc (10 mM) was added into the spray solution for all experiments.

Furthermore, we found that InESI-CID-MS protocol could rapidly discover backbone sequence of peptide both beyond and within DSB with slight modification of conventional nanoESI, as demonstrated by the “cleavage ON/OFF” experiment (Fig. 2a). Firstly, in “cleavage OFF” mode (Fig. 2a “OFF”), conventional nanoESI-CID-MS analysis of GSSG was conducted and peptide sequences containing intact DSB were obtained (Fig. 2b). Then, in “cleavage ON” mode (Fig. 2a “ON”), GSSG was reacted with $\text{HO}\cdot$ and peptide sequences from DSB dissociation was obtained (Fig. 2c). The “cleavage ON” and “cleavage OFF” modes could be swiftly and easily switched (Fig. 2a). Comparison of their tandem MS spectra enables identification of either intact or DSB-cleaved peptide, with the same solution and spray emitter. The above results implied this protocol would be an informative tool for DSB protein analysis, such as exploring the number and location of DSB as well as concomitant backbone sequence information within DSB.

DSB cleavage in InESI-CID-MS is attributed to the selective reaction between DSB and $\text{HO}\cdot$, since tandem MS spectra of the product ions showed almost identical pathways to previous study.³⁴ We also assume that the in situ generation of $\text{HO}\cdot$ correlates with the enhanced corona discharge originated from the regulation of pulsed high voltage in InESI. To validate these assumptions, our investigation was performed in three steps: 1) exploring the relationship between DSB cleavage and pulsed high voltage, 2) characterizing the relationship between pulsed high voltage and corona discharge and 3) proving the relationship between DSB cleavage and corona discharge.

In order to explore the relationship between DSB cleavage and pulsed high voltage, GSSG (including an interchain DSB) was chosen as a model compound. Various amplitudes of pulsed high voltage were used to analyze 8 μM GSSG in water solution. With increased amplitude of the pulsed high voltage, increased intensities of the cleavage products ($\text{GSO}\cdot$ and GSH) were observed (Fig. 3a). GSH and $\text{GSO}\cdot$ are the pair products from DSB dissociation, as shown in the possible cleavage pathway (Scheme S1). Cascade oxidation products of GSH were also observed (Fig. 3a), as GSH could be easily oxidized. The ratio of $\text{GSO}\cdot$ to GSSG increased with elevation of the pulsed high voltage amplitude (Fig. 3b). Similar trend was observed with the ratio of GSH to GSSG (Fig. S3). It should be noted that the increase amplitudes of GSH and $\text{GSO}\cdot$ showed distinct difference. Presumably, it is mainly because the short-lived $\text{GSO}\cdot$ is not easily observed in mass spectrometer. The effect of frequency on DSB cleavage showed similar trend with that of amplitude of the pulsed spray voltage. (Fig. S4). Similar DSB dissociations were also found for other DSB-containing peptides (selectin binding peptide and somatostatin). The above observations indicated that DSB of GSSG was increasingly dissociated with increased amplitude of the pulsed high voltage. Moreover, the presence of volatile electrolyte (NH_4OAc or NH_4HCO_3) of low concentration (<10 mM) in spray solution would greatly facilitate the DSB dissociation (Fig. S5).

Relationship between pulsed high voltage and corona discharge was also characterized. Although $\text{HO}\cdot$ from corona discharge is assumed to be responsible for DSB cleavage in this study, its presence in this system cannot be verified by MS detection. Thus we measured the electrospray-based light emission (ESL), a known sign of corona discharge³⁶, to evaluate the corona discharge during InESI. In a dark room, a PMT was placed under the spray tip to measure the ESL (Inset in Fig. 3c), which is in accordance with previous investigation on corona discharge in low current (< 0.2 μA) ESI.³⁶ ESL signals increased in accordance with the elevation of the amplitude of pulsed high voltage, indicating corona discharge is enhanced gradually (Fig. 3b). Thus, the relationship between pulsed

high voltage and corona discharge in InESI could be directly characterized by measuring the ESL with a PMT.

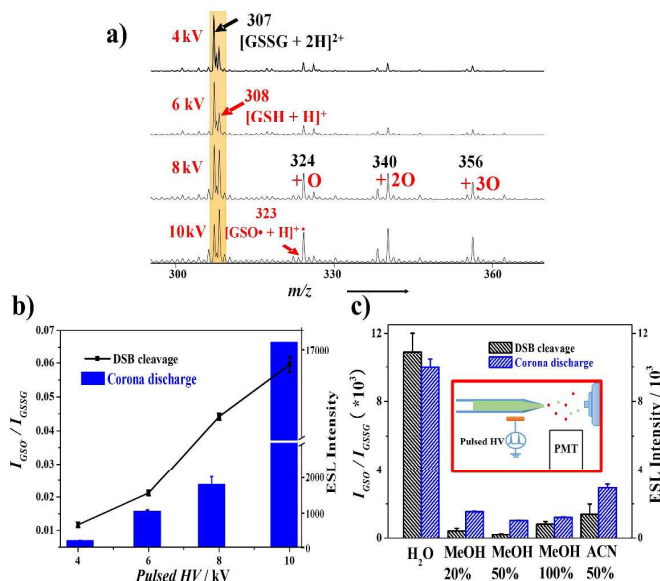
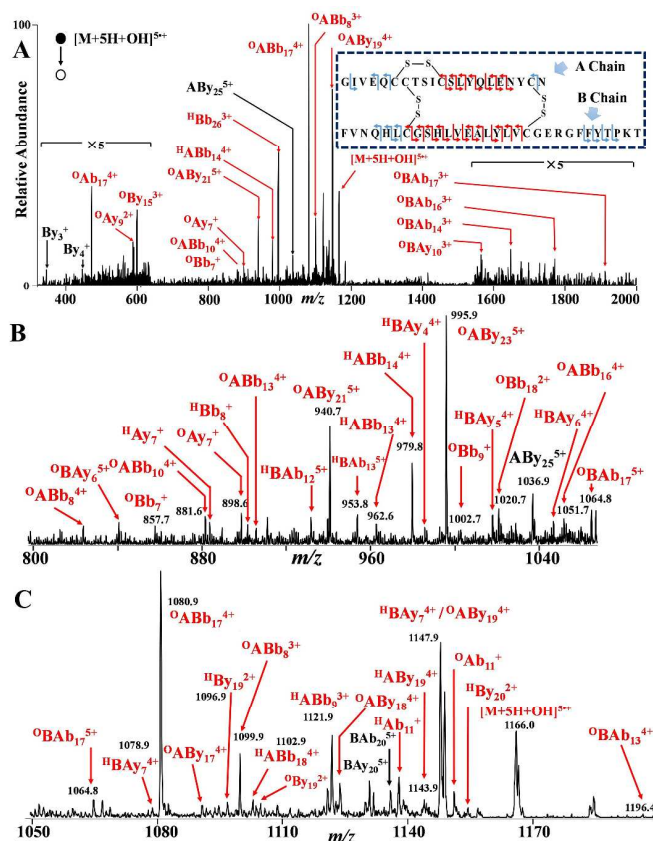


Fig. 3 Dependence of oxidative cleavage reaction on corona discharge. a) Effect of the amplitude of the pulsed high voltage (1 kHz) on disulfide cleavage of GSSG (8 μ M). b) Relationship between DSB cleavage and corona discharge. c) Solvent-effect on DSB cleavage in InESI (pulse, 1 kHz, 6 kV). Inset in c) shows the characterization of corona discharge in InESI with a photomultiplier tube (PMT). In all these experiments, NH₄OAc (5 mM) was added into the spray solution. For the identification of cleavage products, especially for [GSO• + H]⁺, CID MS/MS (Fig. 2c) was used and thus isotopic interfere from near peaks can be avoided. For all the characterization of cleavage degree, intact GSSG signal with minimal reaction (4 kV) was used as the denominator to make a relative fair compare.

We then turned to investigate the relationship between DSB cleavage and corona discharge. In regard to solvent-effect, significant radical-induced DSB cleavage could be observed only in pure H₂O solution, addition of organic solvents would result in dramatic decreasing of the cleavage product (Fig. 3c). That might be attributed to the organic solvents used in our system (methanol and acetonitrile) might also serve as electron scavengers and thus hinder enough corona discharge for HO• generation. Therefore, weak corona discharge would be found for organic solvents even with the presence of additional electrolyte but strong corona discharge for pure H₂O solution. As a result, the radical induced cleavage of DSB could only be observed in H₂O solution, which facilitates corona discharge generation. Except for solvent-effect, the effect of pulsed high voltage amplitude on DSB cleavage was also compared in the aspect of corona discharge. The increase trend for the dependence of DSB cleavage on the amplitude of pulsed high voltage (Fig. 3a/b) was well in accordance with that of corona discharge (Fig. 3b), indicating corona discharge is related with amplitude of the pulsed high voltage and DSB cleavage. Furthermore, to exclude the possibility of DSB cleavage prior to electrospray, sonic spray ionization (free from corona discharge due to the absence of high voltage) was introduced as a control experiment and no obvious cleavage product ions (GSH or GSO•) were observed (Fig. S6). In short, DSB cleavage via oxidative cleavage reaction in InESI is closely related to the existence of corona discharge.



that of ECD (49%)⁴⁷ and ETD (64%)⁴⁸. Thus, extensive sequence information of DSB-linked native protein could be revealed by InESI-CID-MS protocol.

In summary, InESI was utilized to generate HO• for selective and in situ cleavage of DSBs for peptides and native proteins. Selective reaction between HO• (generated from corona discharge) and DSB is the key process in InESI-CID-MS protocol. Peptide sequence of in-between and beyond DSB could be successively distinguished simply by regulating the amplitude of induced high voltage in InESI-CID-MS protocol or by rapid switch between InESI and nanoESI. Although the reaction yield and the HO•-induced disulfide scrambling should be treated with caution, the representative characteristics of InESI-CID-MS protocol are: i) free of additional tedious reducing steps (in situ DSB dissociation) or additional devices for HO• generation (low temperature plasma) that should avoid the time- and sample-consuming wet chemistry, ii) native and rapid sequencing of DSB-bridged peptides/proteins with extensive amino acid sequence information that would facilitate the high throughput application, which are vital for potential applications in the realm of proteomics.

In typical InESI-CID-MS experiments, the size of spray emitter was kept ~5 μm. All experiments were carried out with a LTQ-Velos Pro (Thermo Fisher Scientific, CA, USA) mass spectrometer. Insulin (recombinant human, CAS no. 11061-68-0) was purchased from J&K Scientific Ltd. (Beijing, China). DTT, GSSG, selectin binding peptide and somatostatin were supplied by Sangon Biotech Co., Ltd (Shanghai, China). Other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). No further purifications were performed for all reagents. Chemical reduction was performed by incubating the native protein/peptide standards at 37 °C with 10 times excess of DTT at pH 7 for 4 hours. All solvents used in this study were of HPLC grade. Purified water was obtained from Milli-Q® Reference System (Millipore Corp., USA) with conductivity of 18.2 MΩ.cm. More detailed instrumental parameters seen in Electronic Supplementary Information.

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Notes and references

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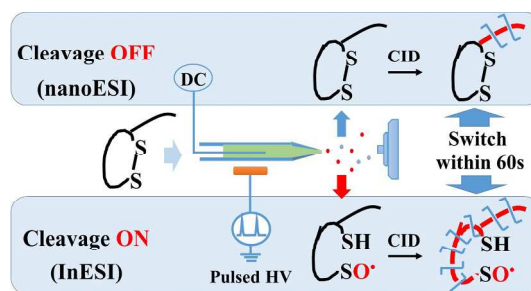
† Electronic Supplementary Information (ESI) available: See DOI: 10.1039/c000000x/

- V. Zabolov, Y. Ge, J. Schwartz and J. W. Walker, *Mol. Cell. Proteomics*, 2008, 7, 1838-1849.
- Y. Ge, I. N. Rybakova, Q. Xu and R. L. Moss, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, 106, 12658-12663.
- H. Zhang and Y. Ge, *Circ Cardiovasc Genet.*, 2011, 4, 711.
- Y.-C. Wang, S. E. Peterson and J. F. Loring, *Cell Res.*, 2014, 24, 143-160.
- J. M. Thornton, *J. Mol. Biol.*, 1981, 151, 261-287.
- T. E. Creighton, *Bioessays*, 1988, 8, 57-63.
- C. N. Pace, G. R. Grimsley, J. A. Thomson and B. J. Barnett, *J. Biol. Chem.*, 1988, 263, 11820-11825.
- M. Matsumura, G. Signor and B. W. Matthews, *Nature*, 1989, 342, 291-293.
- W. J. Wedemeyer, E. Welker, M. Narayan and H. A. Scheraga, *Biochemistry*, 2000, 39, 4207-4216.
- A. Bindoli and M. P. Rigobello, *Antioxid. Redox Signal.*, 2013, 18, 1557-1593.
- J. A. Loo, C. G. Edmonds, H. R. Udseth and R. D. Smith, *Anal. Chem.*, 1990, 62, 693-698.

- X. Han, M. Jin, K. Breuker and F. W. McLafferty, *Science*, 2006, 314, 109-112.
- G. Wang and I. A. Kaltashov, *Anal. Chem.*, 2014, DOI: 10.1021/ac501789e.
- M. Cammarata, K. Y. Lin, J. Pruet, H. W. Liu and J. Brodbelt, *Anal. Chem.*, 2014, 86, 2534-2542.
- L. Sun, G. Zhu, Y. Zhao, X. Yan, S. Mou and N. J. Dovichi, *Angew. Chem., Int. Edit.*, 2013, 52, 13661-13664.
- G. Zhu, L. Sun, X. Yan and N. J. Dovichi, *Anal. Chem.*, 2014, DOI: 10.1021/ac5004486.
- M. S. Lowenthal, Y. Liang, K. W. Phinney and S. E. Stein, *Anal. Chem.*, 2014, 86, 551-558.
- X. Zhang, E. Y. T. Chien, M. J. Chalmers, B. D. Pascal, J. Gatchalian, R. C. Stevens and P. R. Griffin, *Anal. Chem.*, 2010, 82, 1100-1108.
- X. Yan, H. Zhang, J. Watson, M. I. Schimerlik and M. L. Deinzer, *Protein Sci.*, 2002, 11, 2113-2124.
- J. A. Burns, J. C. Butler, J. Moran and G. M. Whitesides, *J. Org. Chem.*, 1991, 56, 2648-2650.
- J. L. Stephenson, B. J. Cargile and S. A. McLuckey, *Rapid Commun. Mass Spectrom.*, 1999, 13, 2040-2048.
- S. Mysling, R. Salbo, M. Ploug and T. J. D. Jørgensen, *Anal. Chem.*, 2014, 86, 340-345.
- H. Permentier and A. Bruins, *J. Am. Soc. Mass Spectrom.*, 2004, 15, 1707-1716.
- S. Nicolardi, A. M. Deelder, M. Palmblad and Y. E. M. van der Burgt, *Anal. Chem.*, 2014, DOI: 10.1021/ac500383c.
- Y. Zhang, H. D. Dewald and H. Chen, *J. Proteome Res.*, 2011, 10, 1293-1304.
- R. A. Zubarev, N. L. Kelleher and F. W. McLafferty, *J. Am. Chem. Soc.*, 1998, 120, 3265-3266.
- H. Oh, K. Breuker, S. K. Sze, Y. Ge, B. K. Carpenter and F. W. McLafferty, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, 99, 15863-15868.
- J. E. P. Syka, J. J. Coon, M. J. Schroeder, J. Shabanowitz and D. F. Hunt, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, 101, 9528-9533.
- D. F. Clark, E. P. Go and H. Desaire, *Anal. Chem.*, 2012, 85, 1192-1199.
- S. D. Patterson and V. Katta, *Anal. Chem.*, 1994, 66, 3727-3732.
- Y. Fukuyama, S. Iwamoto and K. Tanaka, *J. Mass Spectrom.*, 2006, 41, 191-201.
- H. Chen, L. S. Eberlin and R. G. Cooks, *J. Am. Chem. Soc.*, 2007, 129, 5880-5886.
- Y. M. Fung, F. Kjeldsen, O. A. Silivra, T. W. Chan and R. A. Zubarev, *Angew. Chem., Int. Edit.*, 2005, 44, 6399-6403.
- Y. Xia and R. G. Cooks, *Anal. Chem.*, 2010, 82, 2856-2864.
- C. A. Stinson and Y. Xia, *Analyst*, 2013, 138, 2840-2846.
- I. L. Kanev, A. Y. Mikheev, Y. M. Shlyapnikov, E. A. Shlyapnikova, T. Y. Morozova and V. N. Morozov, *Anal. Chem.*, 2014, 86, 1511-1517.
- G. Huang, G. Li and R. G. Cooks, *Angew. Chem., Int. Edit.*, 2011, 50, 9907-9910.
- G. Li and G. Huang, *J. Mass Spectrom.*, 2014, 49, 639-645.
- W. N. English, *Phys. Rev.*, 1948, 74, 179-189.
- G. A. Dawson, *J. Geophys. Res.*, 1969, 74, 6859-6868.
- J. Lloyd and S. Hess, *J. Am. Soc. Mass Spectrom.*, 2010, 21, 2051-2061.
- A. N. Vilkov, V. V. Laiko and V. M. Doroshenko, *J. Mass Spectrom.*, 2009, 44, 477-484.
- J. Lloyd and S. Hess, *J. Am. Soc. Mass Spectrom.*, 2009, 20, 1988-1996.
- M. Chen and K. D. Cook, *Anal. Chem.*, 2007, 79, 2031-2036.
- S. D. Maleknia, M. R. Chance and K. M. Downard, *Rapid Commun. Mass Spectrom.*, 1999, 13, 2352-2358.
- P. A. Chrisman and S. A. McLuckey, *J. Proteome Res.*, 2002, 1, 549-557.
- R. A. Zubarev, N. A. Kruger, E. K. Fridriksson, M. A. Lewis, D. M. Horn, B. K. Carpenter and F. W. McLafferty, *J. Am. Chem. Soc.*, 1999, 121, 2857-2862.
- J. Liu, H. P. Gunawardena, T.-Y. Huang and S. A. McLuckey, *Int. J. Mass Spectrom.*, 2008, 276, 160-170.

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Disulfide peptide sequence coverage could be increased via switching “cleavage ON/OFF” modes between conventional and induced nanoelectrospray MS within 1 min.