© American Society for Mass Spectrometry, 2016

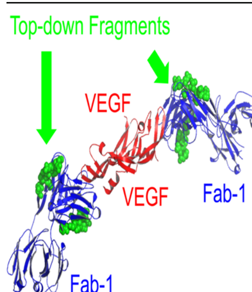
J. Am. Soc. Mass Spectrom. (2016) 27:1139–1142
DOI: 10.1007/s13361-016-1398-9

SHORT COMMUNICATION

Native MS and ECD Characterization of a Fab–Antigen Complex May Facilitate Crystallization for X-ray Diffraction

Ying Zhang,[1,3] Weidong Cui,[1] Aaron T. Wecksler,[2] Hao Zhang,[1] Patricia Molina,[2] Galahad Deperalta,[2] Michael L. Gross[1]

[1]Department of Chemistry, Washington University in St. Louis, One Brookings Dr., St. Louis, MO 63130, USA
[2]Protein Analytical Chemistry, Genentech, a Member of the Roche Group, South San Francisco, CA 94080, USA
[3]*Present Address:* Analytical Research and Development, Pfizer Inc., Chesterfield, MO 63017, USA



Abstract. Native mass spectrometry (MS) and top-down electron-capture dissociation (ECD) combine as a powerful approach for characterizing large proteins and protein assemblies. Here, we report their use to study an antibody Fab (Fab-1)–VEGF complex in its near-native state. Native ESI with analysis by FTICR mass spectrometry confirms that VEGF is a dimer in solution and that its complex with Fab-1 has a binding stoichiometry of 2:2. Applying combinations of collisionally activated dissociation (CAD), ECD, and infrared multiphoton dissociation (IRMPD) allows identification of flexible regions of the complex, potentially serving as a guide for crystallization and X-ray diffraction analysis.

Keywords: Fourier transform ICR mass spectrometry, Native MS, Electron capture dissociation (ECD), X-ray crystallography, High-order structure (HOS), Fab-Antigen Complex, VEGF

Received: 15 March 2016/Revised: 22 March 2016/Accepted: 23 March 2016/Published Online: 21 April 2016

Introduction

Methods are increasingly needed to characterize protein therapeutics, including monoclonal antibodies (mAbs) and their complexes. Although low-resolution circular dichroism and size-exclusion chromatography, complemented by high-resolution nuclear magnetic resonance (NMR) and X-ray crystallography, provide solutions [1–3], they are labor-intensive, require large sample size, and sometimes are restricted in protein size. Thus, the need increases to provide rapid approaches to high order structure (HOS).

MS can determine the molecular weight and primary structure of an antibody-antigen complex [4, 5], but more structural information can be obtained utilizing bottom-up or top-down analysis. Using the former, one can obtain peptide and even the residue-level data through protein footprinting by hydrogen deuterium exchange [6, 7], hydroxyl radical, or other labeling reactions [8–12], yielding maps of epitope binding and complementarity-determining regions (CDR).

Although a top-down *structural* approach with native MS affords high sensitivity, fast turn-around, and no artifacts from digestion [13–15], fragmentation to give high-order structure is difficult. The coarse-grained information from this approach identifies subunits, post-translational modifications (PTMs), and metal binding sites [16]. CAD enables good desolvation, ECD promotes backbone cleavages to identify flexible regions, and IRMPD releases fragments that had remained bound after ECD.

Results and Discussion

Making the reasonable assumption that native ESI allows a protein complex to maintain much of its native structure in the gas phase, we applied ECD, coupled with CAD and IRMPD to take apart a Fab-antigen complex. The structural information obtained from ECD pinpoints surface/flexible regions of the complex [17–23], providing information on the epitope or CDR. If the fragmentation occurs in flexible, surface regions, as we have hypothesized, those regions that deter crystallization or are difficult to analyze by either NMR or X-ray crystallography become identifiable. The surface/flexible regions can either be within the complex or within the individual proteins. This information is alternatively available by limited

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1398-9) contains supplementary material, which is available to authorized users.

Correspondence to: Michael L. Gross; *e-mail:* mgross@wustl.edu

proteolysis [24–26], but that requires longer preparation times and larger amounts of samples.

The system for study is an antibody Fab (Fab-1) and its antigen, VEGF, a highly specific angiogenic factor that stimulates vasculogenesis and angiogenesis [27–29]. The Fab-1 region originates from a neutralizing humanized mAb A4.6.1. We chose this system because this type is of high interest in biotechnology and the pharmaceutical industry, and there is a crystal structure to allow comparison of results. Our objectives are to determine stoichiometry and to inquire whether flexible and dynamic regions of the complex can be located by ECD. Given that these flexible regions often stand in the way of crystallization, we are asking whether ECD information can direct modifications of the protein to facilitate crystallization for X-ray structure determination.

We contrast the outcome of introducing the Fab-1–VEGF complex into a mass spectrometer by denaturing ESI for MW measurement and by native MS to confirm that VEGF in the complex is a covalently linked dimer in solution (Supplementary Figure S1). Denaturing ESI gives a better measurement of MW than does native MS because more solvent and salts may remain attached during the latter. Native MS preserves, at least in part, the high-order structure seen in the solid-state X-ray structure [30, 31]. From native MS, we learned the complex has a MW of 135.12 kDa and contains one VEGF dimer (monomer MW = 19.17 kDa) and two Fab-1 proteins (monomer MW = 48.40 kDa). Thus, the stoichiometry of the complex is 2:2 Fab-1:VEGF (Figure 1a; native MS of Fab-1 is in Supplementary Figure S2). Although the outcome is consistent with a previously published X-ray structure of the complex [32], native MS reveals the stoichiometry of the noncovalent complex in solution with lower sample quantity than X-ray, higher accuracy than SEC and multi-angle light scattering (MALS), and faster turnaround than most other biophysical approaches [33–35].

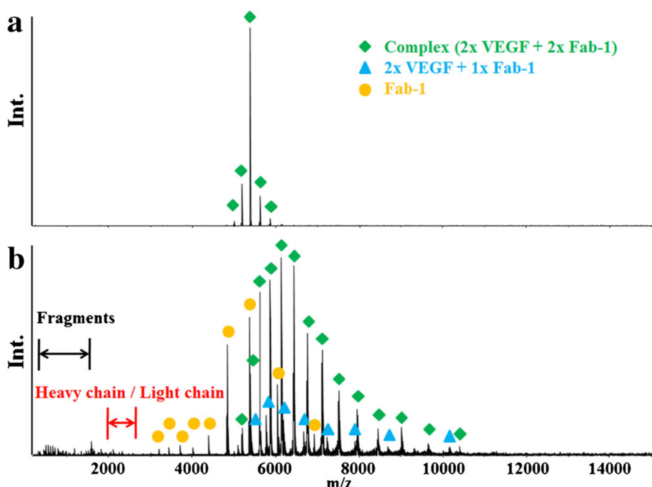


Figure 1. Native MS (**a**) and (**b**) product-ion spectrum of the complex produced by combining CAD, ECD, and IRMPD. "Heavy Chain/Light Chain" highlighted regions show peaks corresponding to the mass of heavy chain (HC) and light chain (LC) from Fab-1. "Fragments" highlighted the region of fragment ions from both VEGF and Fab-1

As expected, the ions from native MS are at high *m/z* (5000 to 6000, from +23 to +27, Figure 1a). These data contrast significantly with the outcome of denaturing ESI for the proteins in their free forms separately, where the median charge states for the heavy chain, light chain, and VEGF are +24, +22, +23, respectively (Supplementary Figures S3–S5). The ions from native MS of the complex are less charged (+23 to +27) than those produced by denatured ESI and exist as a much smaller distribution. Native ESI preserves the tertiary structure of the complex, at least on the short timescale of MS, and introduces a stable complex that retains sufficient high-order structure to maintain the complex intact [15], whereas denaturing ESI breaks noncovalent interactions.

Successful introduction into a mass spectrometer affords the opportunity to apply MS/MS methods to the protein complex. We previously reported that a combination of CAD, ECD, and IRMPD does fragment constituents in flexible regions rather than dissociate the complex into constituent proteins as does CAD on quadrupole time-of-flight mass spectrometers (Q-ToF) [17]. These data can serve as a guide to modify antibody-antigen complexes for X-ray diffraction analysis.

The dissociation methods produce several species, as seen in the product-ion spectrum of the complex (Figure 1b and Table 1). A dominant dissociation is to charge-reduced ions in the higher *m/z* region (i.e., lower charge states) than those of the precursor. Although these reveal little about structure, several ions within this *m/z* region can map the Fab-1–VEGF interaction. The top-down mass spectrum shows formation of ions with masses of the complex minus one Fab-1 (2 VEGF + 1 Fab-1) and of the Fab-1 alone (Figure 1), indicating step-wise dissociation of the Fab-1 from the complex. The process whereby both Fab-1 molecules are expelled from the complex is not dominant. More importantly, our results indicate that in the complex, each Fab-1 protein interacts with one core dimeric VEGF, as shown in the X-ray structure [32]. In addition, we observe ions that represent the heavy chain (HC) and the light chain (LC) from the Fab-1. There is only one inter-chain disulfide bond between HC and LC; thus, the structure of Fab-1 is easier to dissociate upon activation than is the full antibody. The outcome provides confirmation that the Fab-1 lies on the perimeter of the complex (all the information from the subunits is summarized in Table 1).

The product ions exist in the low *m/z* region and provide additional structural detail. We identified three series of fragment ions from three distinct species, the HC and LC from the Fab-1 (Figure 2a), and VEGF (Figure 2b). We observed c_3-c_{10} and z_2-z_7 ions from HC (E1 to G10 and S225 to L231, respectively), c_3-c_9 (except c_7 because the proline-8 of the Fab-1 LC cannot be cleaved) from LC (D1 to S9), and c_3-c_{12} from VEGF (A1 to H12). Some of the small c and z ions occur in congested region and are difficult to identify. In addition to these c and z ions, there are several b and y ions from both HC (b_4 and y_4, L4 and K228, respectively) and LC (b_4 to b_7, L4 to S7) likely formed in IRMPD (Supplementary Figure S6).

Table 1. Identified Components in Native ESI Upon Activation of the Fab-1–VEGF Complex by CAD, ECD, and IRMPD

Protein	*m/z*	Deconvolved aver. mass (theoretical) in kDa
Intact complex 2 VEGF + 2 Fab-1	5000–10,000	135.12 (135.11)
2 VEGF + 1 Fab-1	5500–8000	86.72 (86.72)
Fab-1	3000–7000	48.40 (48.39)
HC and LC of Fab-1	1900–2500	24.96 (24.96); 23.44 (23.43)
Fragments	200–1300	see Figure 2

Although these fragments delineate part of the sequences of Fab-1 and VEGF, they also indicate that the corresponding regions either lie on the surface and/or are highly flexible, and can be excluded from any binding interface. Indeed, the crystal structure indicates that these regions do not include the CDR or epitope-binding residues.

In X-ray crystallography, flexible regions of a protein often hamper crystallization and are candidates for removal to facilitate crystallization. Prior identification of such flexible regions relies on limited proteolysis by nonspecific or combinations of specific proteolytic enzymes, requiring time and large quantity of sample [36]. Native ESI and activation can provide similar information from a single experiment, with low sample consumption (μg) and fast turnaround (up to 2 h).

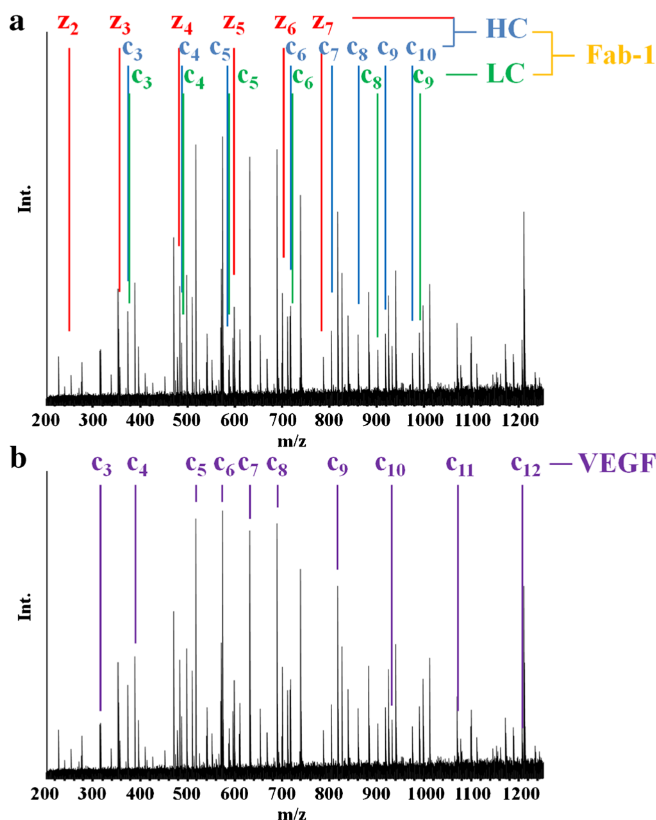


Figure 2. Zoom-in region of the native MS upon activation by combination of CAD, ECD, and IRMPD (Figure 1b) of *m/z* 200–1250. The assigned fragments (c and z ions) are highlighted in different colors according to their source. The unassigned peaks may represent internal fragments, which cannot be identified by current software

Some of the fragment ions represent surface residues (c_3 to c_{10} for HC, and c_3–c_9 for LC), but most of them are from regions either not seen in the X-ray structure or deleted to obtain the crystals. For example, of the 231 residues of the HC, the X-ray structure reveals only residues from E1 to K224. The ECD of Fab-1 gives z_2–z_7 ions from the HC (Figure 2a), indicating that region S225–L231 is highly flexible, dynamic, and appropriate to remove for X-ray crystallography. Similarly, the receptor-binding domain of VEGF that was included for X-ray crystallography began at G8, but the structure is seen starting at E13, indicating that the first 12 residues (A1 to H12) are highly flexible and do not diffract well. Indeed, we observe abundant c_3 to c_{12} ions (Figure 2b), corresponding to residues A1 to H12 of VEGF. Thus, those regions of the C-terminus of the Fab-1 HC and the N-terminus of VEGF fragment readily, but they do not show in the X-ray structure.

We emphasize that this approach contrasts significantly with top-down approaches using denaturing electrospray, where the protein charge is higher and the fragmentation is more efficient as needed for sequencing. Denaturing ESI was previously used for mAbs [13, 37, 38], where the goal was high sequence coverage. Our strategy is to keep together the complex by native MS to provide binding stoichiometry, flexible-region location, and even partial sequence, which cannot be obtained following denaturing ESI.

Methods

Methods and instrumentation are described in detail in the Supplementary Information. The Fab-1–VEGF complex was prepared with a 2:1 molar ratio, purified by size exclusion chromatography, and re-analyzed to ensure purity. The proteins were buffer-exchanged into 200 mM NH_4OAc (pH 6.8), centrifuged, and washed to complete desalting.

Native MS and activation were on a Bruker SolariX 12 T FT-ICR, as described previously [16]. All products were produced by a combination of CAD, ECD, and IRMPD in series. Denaturing ESI, as described previously [7], followed sample denaturation with urea, reduction with dithiotheritol (DTT), acidification with HCOOH, and separation on a C4 analytical column prior to MS analysis in the positive-ion mode of a MaXis 4G quadrupole time-of-flight mass spectrometer (Bruker, Bremen, Germany). Product ions were identified by Biotools software; assignments were accepted if experimental *m/z* was within 5 ppm of theory, had a signal-to-noise ratio greater than 5:1, and were validated manually.

Acknowledgments

Authors thank Victor Ling for helpful discussions, the National Institute of General Medical Sciences of the NIH (grant P41GM103422) and the Protein Analytical Chemistry Group of Genentech for support.

References

1. Lundblad, R.L.: Introduction to biopharmaceutical conformational analysis. In: Approaches to the conformational analysis of biopharmaceuticals; Chapman and Hall/CRC: pp. 1–18 (2009)
2. Razinkov, V.I., Treuheit, M.J., Becker, G.W.: Methods of high throughput biophysical characterization in biopharmaceutical development. Curr. Drug Discov. Technol. **10**, 59–70 (2013)
3. Das, T.K.: Biophysical methods for biotherapeutics: Discovery and development applications; John Wiley and Sons, Inc.: (2014)
4. Beck, A., Sanglier-Cianferani, S., Van Dorsselaer, A.: Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry. Anal. Chem. **84**, 4637–4646 (2012)
5. Zhang, Z., Pan, H., Chen, X.: Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom. Rev. **28**, 147–176 (2009)
6. Houde, D., Engen, J.R.: Conformational analysis of recombinant monoclonal antibodies with hydrogen/deuterium exchange mass spectrometry. Methods Mol. Biol. (Clifton, NJ) **988**, 269–289 (2013).
7. Zhang, Y., Rempel, D.L., Zhang, J., Sharma, A.K., Mirica, L.M., Gross, M.L.: Pulsed hydrogen-deuterium exchange mass spectrometry probes conformational changes in amyloid beta (abeta) peptide aggregation. Proc. Natl. Acad. Sci. U S A **110**, 14604–14609 (2013)
8. Deperalta, G., Alvarez, M., Bechtel, C., Dong, K., McDonald, R., Ling, V.: Structural analysis of a therapeutic monoclonal antibody dimer by hydroxyl radical footprinting. mAbs **5**, 86–101 (2013)
9. Zhang, Y., Rempel, D.L., Zhang, H., Gross, M.L.: An improved fast photochemical oxidation of proteins (fpop) platform for protein therapeutics. J. Am. Soc. Mass Spectrom. **26**, 526–529 (2015)
10. Kaur, P., Tomechko, S., Kiselar, J., Shi, W., Deperalta, G., Wecksler, A.T., Gokulrangan, G., Ling, V., Chance, M.R.: Characterizing monoclonal antibody structure by carbodiimide/gee footprinting. mAbs **6**, 1486–1499 (2014)
11. Kaur, P., Tomechko, S.E., Kiselar, J., Shi, W., Deperalta, G., Wecksler, A.T., Gokulrangan, G., Ling, V., Chance, M.R.: Characterizing monoclonal antibody structure by carboxyl group footprinting. mAbs **7**, 540–552 (2015)
12. Wecksler, A.T., Kalo, M.S., Deperalta, G.: Mapping of fab-1:VEGF interface using carboxyl group footprinting mass spectrometry. J. Am. Soc. Mass Spectrom. **26**, 2077–2080 (2015)
13. Beck, A., Wagner-Rousset, E., Ayoub, D., Van Dorsselaer, A., Sanglier-Cianferani, S.: Characterization of therapeutic antibodies and related products. Anal. Chem. **85**, 715–736 (2013)
14. Breuker, K., Bruschweiler, S., Tollinger, M.: Electrostatic stabilization of a native protein structure in the gas phase. Angew. Chem. Int. Ed. Engl. **50**(873–877) (2011)
15. Benesch, J.L., Robinson, C.V.: Mass spectrometry of macromolecular assemblies: preservation and dissociation. Curr. Opin. Struct. Biol. **16**, 245–251 (2006)
16. Zhang, H., Cui, W., Wen, J., Blankenship, R.E., Gross, M.L.: Native electrospray and electron-capture dissociation FTICR mass spectrometry for top-down studies of protein assemblies. Anal. Chem. **83**, 5598–5606 (2011)
17. Zhang, H., Cui, W., Wen, J., Blankenship, R.E., Gross, M.L.: Native electrospray and electron-capture dissociation in FTICR mass spectrometry provide top-down sequencing of a protein component in an intact protein assembly. J. Am. Soc. Mass Spectrom. **21**, 1966–1968 (2010)
18. Zhang, H., Cui, W., Gross, M.L.: Mass spectrometry for the biophysical characterization of therapeutic monoclonal antibodies. FEBS Lett. **588**, 308–317 (2014)
19. Lermyte, F., Sobott, F.: Electron transfer dissociation provides higher-order structural information of native and partially unfolded protein complexes. Proteomics **15**, 2813–2822 (2015)
20. Lermyte, F., Konijnenberg, A., Williams, J.P., Brown, J.M., Valkenborg, D., Sobott, F.: ETD allows for native surface mapping of a 150 kDa noncovalent complex on a commercial q-TWIMS-ToF instrument. J. Am. Soc. Mass Spectrom. **25**, 343–350 (2014)
21. Li, H., Wolff, J.J., Van Orden, S.L., Loo, J.A.: Native top-down electrospray ionization-mass spectrometry of 158 kDa protein complex by high-resolution Fourier transform ion cyclotron resonance mass spectrometry. Anal. Chem. **86**, 317–320 (2014)
22. Li, H., Wongkongkathep, P., Van Orden, S.L., Ogorzalek Loo, R.R., Loo, J.A.: Revealing ligand binding sites and quantifying subunit variants of noncovalent protein complexes in a single native top-down fticr ms experiment. J. Am. Soc. Mass Spectrom. **25**, 2060–2068 (2014)
23. Zhang, J., Reza Malmirchegini, G., Clubb, R.T., Loo, J.A.: Native top-down mass spectrometry for the structural characterization of human hemoglobin. Eur. J. Mass Spectrom. (Chichester, Engl.) **21**, 221–231 (2015).
24. Dong, A., Xu, X., Edwards, A.M., Chang, C., Chruszcz, M., Cuff, M., Cymborowski, M., Di Leo, R., Egorova, O., Evdokimova, E., Filippova, E., Gu, J., Guthrie, J., Ignatchenko, A., Joachimiak, A., Klostermann, N., Kim, Y., Korniyenko, Y., Minor, W., Que, Q., Savchenko, A., Skarina, T., Tan, K., Yakunin, A., Yee, A., Yim, V., Zhang, R., Zheng, H., Akutsu, M., Arrowsmith, C., Avvakumov, G.V., Bochkarev, A., Dahlgren, L.G., Dhe-Paganon, S., Dimov, S., Dombrovski, L., Finerty, P., Jr., Flodin, S., Flores, A., Graslund, S., Hammerstrom, M., Herman, M.D., Hong, B.S., Hui, R., Johansson, I., Liu, Y., Nilsson, M., Nedyalkova, L., Nordlund, P., Nyman, T., Min, J., Ouyang, H., Park, H.W., Qi, C., Rabeh, W., Shen, L., Shen, Y., Sukumard, D., Tempel, W., Tong, Y., Tresagues, L., Vedadi, M., Walker, J.R., Weigelt, J., Welin, M., Wu, H., Xiao, T., Zeng, H., Zhu, H.: In situ proteolysis for protein crystallization and structure determination. Nat. Methods **4**, 1019–1021 (2007)
25. Wernimont, A., Edwards, A.: In situ proteolysis to generate crystals for structure determination: an update. PLoS ONE **4**, e5094 (2009)
26. Fontana, A., de Laureto, P.P., Spolaore, B., Frare, E.: Identifying disordered regions in proteins by limited proteolysis. Methods Mol. Biol. (Clifton, NJ) **896**, 297–318 (2012).
27. Dvorak, H.F., Brown, L.F., Detmar, M., Dvorak, A.M.: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. **146**, 1029–1039 (1995)
28. Ferrara, N.: The role of vascular endothelial growth factor in pathological angiogenesis. Breast Cancer Res. Treat. **36**, 127–137 (1995)
29. Folkman, J.: Angiogenesis in cancer, vascular, rheumatoid, and other disease. Nat. Med. **1**, 27–31 (1995)
30. Muller, Y.A., Christinger, H.W., Keyt, B.A., de Vos, A.M.: The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 a resolution: multiple copy flexibility and receptor binding. Structure (London, Engl.) **5**, 1325 (1997).
31. Muller, Y.A., Li, B., Christinger, H.W., Wells, J.A., Cunningham, B.C., de Vos, A.M.: Vascular endothelial growth factor: Crystal structure and functional mapping of the kinase domain receptor binding site. Proc. Natl. Acad. Sci. U S A **94**, 7192–7197 (1997)
32. Muller, Y.A., Chen, Y., Christinger, H.W., Li, B., Cunningham, B.C., Lowman, H.B., de Vos, A.M.: VEGF and the Fab fragment of a humanized neutralizing antibody: crystal structure of the complex at 2.4 a resolution and mutational analysis of the interface. Structure (London, Engl.) **6**, 1153–1167 (1998).
33. Hopper, J.T., Robinson, C.V.: Mass spectrometry quantifies protein interactions-from molecular chaperones to membrane porins. Angew. Chem. Int. Ed. **53**, 14002–14015 (2014)
34. Snijder, J., Heck, A.J.: Analytical approaches for size and mass analysis of large protein assemblies. Annu. Rev. Anal. Chem. (Palo Alto, CA) **7**, 43–64 (2014).
35. Loo, J.A.: Studying noncovalent protein complexes by electrospray ionization mass spectrometry. Mass. Spectrom. Rev. **16**, 1–23 (1997)
36. Kleemann, G.R., Beierle, J., Nichols, A.C., Dillon, T.M., Pipes, G.D., Bondarenko, P.V.: Characterization of IgG1 immunoglobulins and peptide-Fc fusion proteins by limited proteolysis in conjunction with LC-MS. Anal. Chem. **80**, 2001–2009 (2008)
37. Valeja, S.G., Kaiser, N.K., Xian, F., Hendrickson, C.L., Rouse, J.C., Marshall, A.G.: Unit mass baseline resolution for an intact 148 kDa therapeutic monoclonal antibody by Fourier transform ion cyclotron resonance mass spectrometry. Anal. Chem. **83**, 8391–8395 (2011)
38. Jones, L.M., Zhang, H., Cui, W., Kumar, S., Sperry, J.B., Carroll, J.A., Gross, M.L.: Complementary MS methods assist conformational characterization of antibodies with altered S–S bonding networks. J. Am. Soc. Mass Spectrom. **24**, 835–845 (2013)