ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/271162811

Metal displacement and stoichiometry of protein–metal complexes under native conditions using capillary electrophoresis/mass spectrometry

ARTICLE *in* RAPID COMMUNICATIONS IN MASS SPECTROMETRY · SEPTEMBER 2010

Impact Factor: 2.25 · DOI: 10.1002/rcm.4995

CITATIONS
21

READS
8

1 AUTHOR:

Mehdi Moini
George Washington University
72 PUBLICATIONS **1,535** CITATIONS

SEE PROFILE

Available from: Mehdi Moini
Retrieved on: 25 January 2016

RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2010; **24**: 2730–2734
Published online in Wiley Online Library (wileyonlinelibrary.com) DOI: 10.1002/rcm.4702

Metal displacement and stoichiometry of protein-metal complexes under native conditions using capillary electrophoresis/mass spectrometry

Mehdi Moini*
Museum Conservation Institute, Smithsonian Institutions, 4210 Silver Hill Road, Suitland, MD 20746-2863, USA

Received 13 May 2010; Revised 15 July 2010; Accepted 15 July 2010

Increases in the study of protein-metal complexes, as well as in metal displacement in protein-metal complexes under native conditions for optimum catalytic properties in drug research and catalyst design, demands a separation/detection technology that can accurately measure metal displacement and stoichiometry in protein-metal complexes. Both nuclear magnetic resonance (NMR) and X-ray diffraction techniques have been used for this purpose; however, these techniques lack sensitivity. Electrospray ionization mass spectrometry (ESI-MS) using direct infusion offers higher sensitivity than the former techniques and provides molecular distribution of various protein-metal complexes. However, since protein-metal complexes under native conditions usually are dissolved in salt solutions, their direct ESI-MS analysis requires off-line sample clean-up prior to MS analysis to avoid sample suppression during ESI. Moreover, direct infusion of the salty solution promotes non-specific salt adduct formation by the protein-metal complexes under ESI-MS, which complicates the identification and stoichiometry measurements of the protein-metal complexes. Because of the high mass of protein-metal complexes and lack of sufficient resolution by most mass spectrometers to separate non-specific from specific metal-protein complexes, accurate protein-metal stoichiometry measurements require some form of sample clean up prior to ESI-MS analysis. In this study, we demonstrate that capillary electrophoresis/electrospray ionization in conjunction with a medium-resolution (~10 000) mass spectrometer is an efficient and fast method for the measurement of the stoichiometry of the protein-metal complexes under physiological conditions (pH ~7). The metal displacement of Co^{2+} to Cd^{2+}, two metal ions necessary for activation in the monomeric AHL lactonase produced by *B. thuringiensis*, has been used as a proof of concept. **Copyright © 2010 John Wiley & Sons, Ltd.**

Quorum-sensing is the ability of bacteria to communicate and coordinate behavior via signaling molecules called autoinducers.[1,2] Quorum-sensing allows bacteria to function as multicellular organisms, contributing to their pathogenicity. As a result, quorum-sensing pathways have been suggested as potential targets for the development of novel therapeutics.[3] Some of the most active quorum-quenching agents known currently are naturally occurring *N*-acyl-L-homoserine-lactonases (AHLs) from *Bacillus thuringiensis*. These agents function by blocking quorum-sensing-dependent processes in several *in vivo* systems, catalyzing the hydrolytic ring opening of AHLs.[4,5] The monomeric AHL (also known as an autoinducer inactivation enzyme (AiiA)[6]) produced by *B. thuringiensis* belongs to the metallo-B-lactamase superfamily and is purified with two tightly bound metal ions, which are shown to be essential for the activity of the complex.[7,8] Evidence suggests that the metal ions associated with this AiiA are required for proper protein folding and function.[9] A variety of metal ions, including Mn^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+}, have been used to displace the dinuclear metal site of this AiiA to determine the optimum composition for enhanced catalytic properties.[9] Because of the increased interest in metal complexes and metal-ligand interactions in drug research[10] and catalyst design,[11] it is necessary to develop novel detection methods that can detect protein-metal stoichiometry accurately and quickly. Several conventional analytical techniques have been utilized to monitor the metal displacement in protein-metal complexes. Electronic absorption and circular dichroism (CD) have been primarily used to monitor the metal displacement during titration studies.[12,13] Electron paramagnetic resonance spectroscopy and nuclear magnetic resonance (NMR) spectroscopy have also been widely used to study the interactions of metal ions and proteins.[14] Despite a number of advantages, these techniques do not allow for precise determination of the stoichiometry of the complexes involved. Direct infusion electrospray ionization mass spectrometry (ESI-MS) has demonstrated potential for determining the molecular distributions of various protein-metal complexes with increased sensitivity

**Correspondence to*: M. Moini, Museum Conservation Institute, Smithsonian Institutions, 4210 Silver Hill Road, Suitland, MD 20746-2863, USA.
E-mail: MoiniM@SI.edu

Copyright © 2010 John Wiley & Sons, Ltd.

compared to NMR or X-ray diffraction techniques.[15–25] Because ESI is considered to be a gentle ionization process, even weak non-covalent complexes can be analyzed. The combination of ESI with moderately high resolving power mass analyzers, such as quadrupole time-of-flight, allows metal ion binding stoichiometry to be determined by directly measuring the molecular weight of the complex. Fast-atom bombardment (FAB)-MS and ESI-MS have both been used in the past to evaluate binding selectivity of various non-covalent complexes.[26] In comparison, ESI-MS represents a simple and elegant approach for gaining insights into the binding stoichiometry and affinity of these assemblies. Unfortunately, the formation of non-specific metal adducts during ESI can be a severe problem, often leading to binding levels that are dramatically higher than those in bulk solution. Therefore, to analyze protein-metal complexes by ESI-MS, the buffer salts must be removed prior to direct infusion to avoid signal suppression and non-specific interactions of salt metals (such as sodium) with the protein of interest. By interfering with the protein-metal complex of interest, non-specific adduct formation may complicate the ESI spectrum, which can lead to inaccurate protein-metal stoichiometry assignments. Since protein-metal stoichiometry must be measured under physiological conditions, prolonged off-line sample clean-up can denature and dissociate these complexes. It has been shown that using tartrate salt can significantly lower the levels of artifact metal binding.[27] In general, rigorous experimental controls need to be performed to establish that the protein-metal stoichiometry detected under ESI is really the result of in-solution interactions rather than artefactual non-specific association occurring during the ESI process.[28] To avoid off-line sample clean-up, on-line size-exclusion column chromatography (SEC) and rapid in-line desalting (RILED) coupled to ESI-MS have been used. The SEC approach was used to both separate proteins in a complex mixture and exchange buffers prior to the ESI process. However, since SEC separation can take up to 60 min for simple mixtures that do not require separation, RILED provides a much faster high-throughput desalting procedure.[29] Reversed-phase high-performance liquid chromatography (HPLC) has also been proposed for this purpose;[30] however, because of the interactions of the stationary phase, losses of species were unavoidable. This was especially relevant for complexes with partially unfilled coordination sphere containing Cu^{2+} or Ag^{2+} ions, or complexes with Zn^{2+}, the latter because of the metal exchange with other metals (Cd^{2+}, Cu^{2+}) adsorbed on the column. Specifically, the competing effects of the HPLC solvent gradient and the ionization efficiencies of the different complexes must also be taken into consideration.

Since separation in capillary electrophoresis (CE) is based on the difference in mobility of ions in solution under applied electric field, the technique offers a convenient method for the separation of protein-metal complexes prior to ESI-MS.[31] By utilizing CE as a separation technique prior to MS analysis, non-specific salt adducts that are weakly attached to proteins in solution can be separated from the protein-metal complex of interest prior to ESI. CE in conjunction with inductively coupled plasma (ICP)-MS has been used previously for the separation of protein-metal complexes in biological samples.[32–36] The recombinant mouse liver metallothionein Zn_7-complex was also studied using CE/ESI-MS.[37] Recently, we demonstrated that CE/MS of protein complexes under native condition provides accurate stoichiometry for protein-protein and protein-metal complexes of lysed red blood cells by removing the non-specific metal adducts from the complex in a manner that maintains the integrity of the specific protein-metal complex.[38] In this article, the metal displacement of Co^{2+} to Cd^{2+}, two metal ions necessary for activation in the monomeric AHL lactonase, produced by *B. thuringiensis*, has been investigated using sheathless CE/MS to determine exact stoichiometric binding ratios for protein-metal complexes.

EXPERIMENTAL

Preparation of protein-metal complexes

The AHL-lactonase encoded by AiiA from *B. thuringiensis* was prepared by Dr. Pei W. Thomas from the Department of Pharmacy at The University of Texas, Austin, Texas. The original AiiA protein was grown in medium containing $CoCl_2$ to produce a protein-metal complex containing AiiA-$2Co^{2+}$. Different concentrations of $CdCl_2$ (40–400 μM) were mixed with AiiA-$2Co^{2+}$ to produce different ratios (1:1, 10:1, and 100:1) of Cd^{2+}:Co^{2+}. At a ratio of 100:1, most of the AiiA protein was precipitated, perhaps due to non-specific binding of $CdCl_2$ to the protein. These mixtures were incubated at 4°C for 2 days. To remove extraneous buffer salts, the resulting complexes were subjected to centrifugal ultrafiltration clean-up and washed with a buffer of 10 mM ammonium acetate (pH 6.9). Protein-metal complexes were diluted with 10 mM ammonium acetate (pH 6.9) to 10 μM before analysis by CE/MS.

Methods and techniques

All protein-metal complexes were separated online with a P/ACE system MDQ CE instrument (Beckman-Coulter, Fullerton, CA, USA) and analyzed by ESI-MS using a Q-TOF Premier mass spectrometer (Waters, Milford, MA, USA). A porous tip CE/MS sheathless interface was used.[39] A 100 cm long, 30 μm i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used for CE separation. A solution of 10 mM ammonium acetate containing 0.1% polybrene (pH 6.9) was used as the CE background electrolyte (BGE).[40]

All MS analyses were performed in positive ionization mode. The CE inlet was maintained at +30 kV (forward polarity mode) with a constant pressure of 10 psi and electrospray voltage of ~1.6 kV. The Q-TOF was operated under the following component voltage conditions: sampling cone 45, extraction cone 5, ion guide 3, LM resolution 4.5, HM resolution 15, ion energy 3, pre-filter 10, collision energy 2, cell entrance 5, cell exit 20, collision cell 1mL/min., detector 2000 V. The temperature of the counter current nitrogen gas was 120°C. The mass spectrometer was scanned in the m/z range of 2000–4000.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

RCM

```
   1   *   10    *   20    *   30    *   40    *   50
 1 GRISMTVKKLYFIPAGRCMLDHSSVNSALTPGKLLNLPVWCYLLETEEGP
51 ILVDTGMPESAVNNEGLFNGTFVEGQILPKMTEEDRIVNILKRVGYEPDD
101 LLYIISSHLHFDHAGGNGAFTNTPIIVQRTEYEAALHREEYMKECILPHL
151 NYKIIEGDYEVVPGVQLLYTPGHSPGHQSLFIETEQSGSVLLTIDASYTK
201 ENFEDEVPFAGFDPELALSSIKRLKEVVKKEKPIIFFGHDIEQEKSCRVF
251 PEYI
```

Figure 1. Sequence of the autoinducer inactivation enzyme A (AiiA) protein from *B. thuringiensis*.

RESULTS AND DISCUSSION

Study of metal stoichiometry in protein-metal complexes

The AiiA from *B. thuringiensis* (average molecular weight (MW) 28633.75) is known to have quorum-quenching activity in the presence of two bound metal ions. The sequence of the protein AiiA is shown in Fig. 1. The AiiA-metal complex is most stable at a pH ~7 in the presence of buffer salts; however, the presence of this buffer during the ESI process suppresses the ionization of the complex. Recently, we have shown that 10 mM ammonium acetate is a good background electrolyte for CE/MS analysis of protein-protein and protein-metal complexes of red blood cells.[38] To examine if this background electrolyte was also useful for AiiA-metal complexes, CE/ESI-MS of the AiiA-$2Co^{2+}$ complex were performed using 10 mM ammonium acetate at pH values of 4.8 and 6.9 (Figs. 2(A) and 2(B), respectively). As shown, at a pH of 4.8 (Fig. 2(A)), the protein-metal complex dissociated and only the protein was detected. In contrast, at pH 6.9 (Fig. 2(B)), the protein-metal complex remained intact. The deconvoluted spectrum in Fig. 2(B) yielded an average MW of 28751 Da, which corresponds to the AiiA-$2Co^{2+}$ complex. Similar to our previous study to obtain an accurate molecular weight free from the non-specific salt adduct, it was necessary to dilute the sample in a solution of 10 mM ammonium acetate (pH 6.9) before performing CE/MS analysis.

To demonstrate that CE/MS was essential for obtaining the accurate MW of the AiiA-metal complex, direct infusion of the diluted sample (without CE separation) was attempted and compared with the mass spectrum of the sample using CE/ESI-MS. Figure 3 shows the infusion ESI-MS spectra of the AiiA-Co^{2+} complex. As shown in Fig. 3, even though the AiiA-Co^{2+} complex was diluted in ammonium acetate solution before infusion ESI-MS, the mass spectrum of the protein-metal complex still contains a number of unresolved peaks caused by multiple non-specific metal (mostly sodium) adductions. Because the

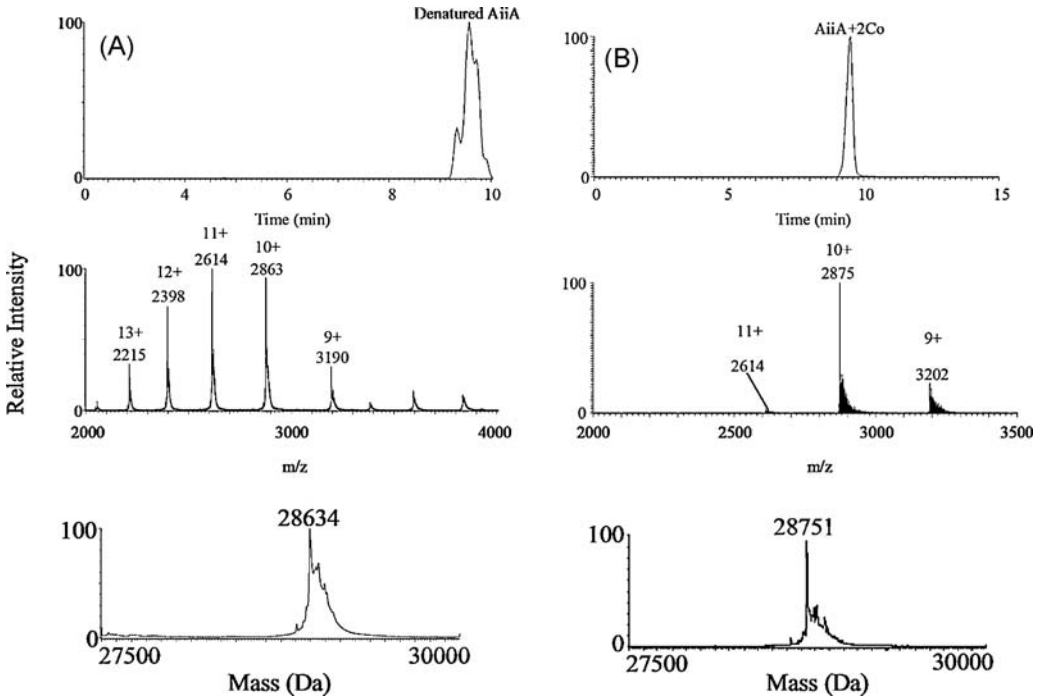

Figure 2. (A) Electropherogram (top panel) and the respective mass spectrum (bottom panel) of the AiiA-$2Co^{2+}$ complex at pH 4.8 using CE/MS. At this pH, the AiiA protein complex is decomposed and no complex with the Co^{2+} is seen. (B) Electropherogram (top panel) and the respective mass spectrum (bottom panel) of the AiiA-$2Co^{2+}$ complex at pH 6.9. At this pH, the AiiA-Co^{2+} complex remains intact.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

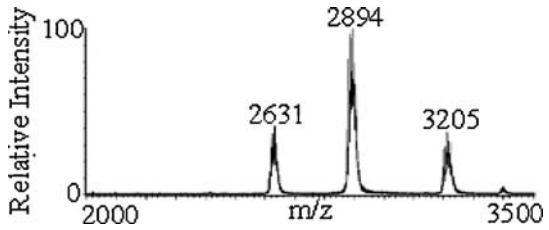

Figure 3. Mass spectrum of the AiiA-2Co^{2+} complex at pH 6.9 obtained using infusion ESI-MS.

resolution of a typical TOF mass analyzer is usually not high enough to differentiate the isotope clusters of protein-metal complexes from proteins bound to non-specific specific metal ions, it becomes difficult to accurately identify the composition and stoichiometry of the protein-metal complexes. Therefore, to utilize direct infusion MS, it is necessary to significantly, but not completely, reduce the salt content by using off-line salt-removal techniques. In contrast, under CE/MS conditions, the mass spectrum of the electrophoretic peak of the same sample contained sharp peaks (Fig. 2(B), middle panel) corresponding to the *m/z* value of the protein-metal complex within the experimental error of ~0.02%.

Study of metal displacement in protein-metal complexes

The AiiA protein was originally grown in a medium containing $CoCl_2$. The mass spectrum of the protein complex shows that the AiiA is binuclear, with two cobalt atoms bound to the protein. To determine the relative binding stoichiometry of the Cd^{2+}, different ratios of $CdCl_2$ (1:1, 10:1, and 100:1) were added to the AiiA-2Co^{2+} complex in order to substitute the two Co^{2+} ions with two Cd^{2+} ions. Figures 4(A)–4(C) show the CE/MS analyses of these three ratios, respectively. The top panels illustrate the electropherogram of the protein-metal complexes, the middle panels show the corresponding mass spectra, and the bottom panels show the deconvoluted mass spectra. From Fig. 4, the relative binding stoichiometry of each metal can be determined (Table 1). The results indicate that, at a 1:1 ratio of Co^{2+} to Cd^{2+}, both Co^{2+} ions are tightly bound to the AiiA protein. However, as more Cd^{2+} is added (10:1 ratio), one Co^{2+} ion is completely replaced by one Cd^{2+} ion. At a 100:1 ratio of Cd^{2+} to Co^{2+}, both Co^{2+} ions are replaced by two Cd^{2+} ions. Because the results were obtained by CE/MS, all free metal ions were separated from the protein-metal complex and did not participate in the complex formation during ESI, allowing a more accurate determination of the ratios of the metal concentration needed for metal displacement.

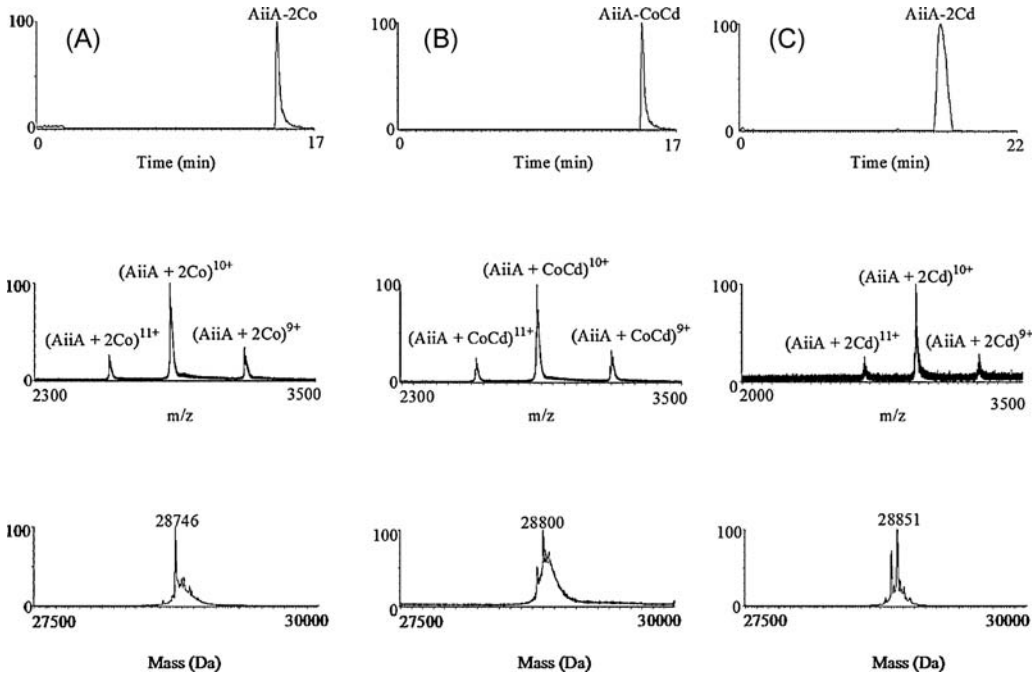

Figure 4. The electropherograms (top panels), mass spectra (middle panels), and deconvoluted mass spectra (bottom panels) of AiiA-metal complexes when a 1:1 ratio (A), a 10:1 ratio (B), and a 100:1 ratio (C) of Cd^{2+}:Co^{2+} solutions were added to the protein solution.

Table 1. Protein-metal stoichiometry at different ratios of Cd^{2+} to Co^{2+}

Ratio	Complex stoichiometry	*m/z* (+9)	*m/z* (+10)	*m/z* (+11)	Measured avg. MW	Calculated avg. MW
1:1	AiiA-2Co^{2+}	2614	2875	3202	28746	28751
10:1	AiiA-$Co^{2+}Cd^{2+}$	2619	2881	3209	28800	28804
1:100	AiiA-2Cd^{2+}	2634	2886	3214	28851	28857

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm

RCM

CONCLUSIONS

We have shown that capillary electrophoresis/electrospray ionization mass spectrometry is a viable technique for determining the stoichiometry of protein-metal complexes, which can provide complementary information to that obtained from circular dichroism, UV-VIS or NMR. By separating loosely attached and non-specific metal ions present in the sample solution, CE/MS minimizes the interference of buffer salts that conventionally hamper direct infusion experiments. Unlike HPLC, CE makes it possible to analyze less stable complexes because the interaction of the complex with the capillary wall is weaker than the chromatographic stationary phase. By utilizing CE as a separation technique prior to ESI-MS analysis, protein-metal ion complexes can readily be observed without interference from salts or extra metal ions present in solution.

Acknowledgements

We wish to thank Ms. Selynda Garza, for helping run the samples and Drs. Pei W. Thomas and Walter Fast from the Department of Pharmacy at The University of Texas at Austin for providing the protein-metal samples.

REFERENCES

1. Parsek MR, Greenber EP. *Trends Microbiol.* 2005; **13**: 27.
2. Dong YH, Zhang LH. *J. Microbiol.* 2005; **43**: 101.
3. Suga H, Smith KM. *Curr. Opin. Chem. Biol.* 2003; **7**: 586.
4. Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH. *Nature* 2001; **411**: 813.
5. Dong YH, Gusti AR, Zhang Q, Xu JL, Zhang LH. *Appl. Environ. Microbiol.* 2002; **68**: 1754.
6. Dong YH, Xu JL, Li XZ, Zhang LH. *Proc. Natl. Acad. Sci. USA* 2000; **97**: 3526.
7. Liu D, Lepore BW, Petsko GA, Thomas PW, Stone EM, Fast W, Ringe D. *Proc. Natl. Acad. Sci. USA* 2005; **102**: 11882.
8. Thomas PW, Stone EM, Costello AL, Tierney DL, Fast W. *Biochemistry* 2005; **44**: 7559.
9. Momb J, Thomas PW, Breece RM, Tierney DL, Fast W. *Biochemistry* 2006; **45**: 13385.
10. Farrell N. *Coord. Chem. Rev.* 2002; **232**: 1.
11. Chen P. *Angew. Chem. Int. Ed.* 2003; **42**: 2832.
12. Cols N, Romero-Isart N, Capdevila M, Olivia B, Gonzalez-Duarte P, Gonzalez-Duarte R, Atrian S. *J. Inorg. Biochem.* 1997; **68**: 157.
13. Capdevila M, Cols N, Romero-Isart N, Gonzalez-Duarte R, Gonzalez-Duarte P. *Cell. Mol. Life Sci.* 1997; **53**: 681.
14. Chaparro AL, Vachet RW. *J. Mass Spectrom.* 2003; **38**: 333.
15. Loo JA. *Int. J. Mass Spectrom.* 2001; **204**: 113.
16. Loo JA. *Mass Spectrom. Rev.* 1997; **16**: 1.
17. Duan G, Wang Y, Aviram M, Swaroop M, Loo JA, Bian J, Tian Y, Mueller T, Bisgaier CL, Sun Y. *Mol. Cell. Biol.* 1999; **19**: 3145.
18. Hu P, Ye QZ, Loo JA. *Anal. Chem.* 1994; **66**: 4190.
19. Veenstra TD, Johnson KL, Tomlinson AJ, Craig R, Kumar R, Naylor S. *J. Am. Soc. Mass Spectrom.* 1998; **9**: 8.
20. Lei QP, Cui X, Kurtz DM Jr, Amster IJ, Chernushevich IV, Standing KG. *Anal. Chem.* 1998; **70**: 1838.
21. Yu X, Wojciechowski M, Fenselau C. *Anal Chem.* 1993; **63**: 1355.
22. Jensen LT, Peltier JM, Winge DT. *J. Bioinorg. Chem.* 1998; **3**: 627.
23. Gwhrig PM, You C, Dallinger R, Gruber C, Brouwer M, Kagi JR, Hunziker PE. *Protein Sci.* 2000; **9**: 395.
24. Stillman MJ, Thomas D, Trevithick C, Guo X, Siu M. *Inorg. Biochem.* 2000; **79**: 11.
25. Traeger JC. *Int. J. Mass Spectrom.* 2000; **200**: 387.
26. Young DS, Hung HY, Liu LK. *J. Mass Spectrom.* 1997; **32**: 432.
27. Pan J, Xu K, Yang X, Wing-Yiu C, Lars K. *Anal. Chem.* 2009; **81**: 5008.
28. Poteir N, Rogniaux H, Chevreux G, Dorsselaer AV. *Methods Enzymol.* 2008; **402**: 361.
29. Benson LM, Kumar R, Cavanagh J, Naylor S. *Rapid Commun. Mass Spectrom.* 2003; **17**: 267.
30. Takatera K, Osaki N, Yamagushi H, Watanabe T. *Anal. Sci.* 1994; **10**: 907.
31. Beattie JH. *Talanta* 1998; **46**: 225.
32. Mounicou S, Polec K, Chassaigne H, Potin-Gautier M, Lobinski R. *J. Anal. At. Spectrom.* 2000; **15**: 635.
33. Polec K, Mounicou S, Chassaigne H, Lobinski R. *Mol. Cell. Biol.* 2000; **46**: 221.
34. Prange A, Schaumloffel D, Bratter P, Richarz A, Wolf C. *Fresenius J. Anal. Chem.* 2001; **372**: 764.
35. Beattie JH, Wood AM, Duncan GJ. *Electrophoresis* 1999; **20**: 1613.
36. Guo X, Chan HM, Guevremont R, Siu KM. *Rapid Commun. Mass Spectrom.* 1999; **13**: 500.
37. Pawlak KP, Palacios O, Capdevila M, Gonzalez-Duarte P, Lobinski R. *Talanta* 2002; **57**: 1011.
38. Nguyen A, Moini M. *Anal. Chem.* 2008; **80**: 7169.
39. Moini M. *Anal. Chem.* 2007; **79**: 4241.
40. Garza S, Chang S, Moini M. *J. Chromatogr. A* 2007; **1159**: 14.

Copyright © 2010 John Wiley & Sons, Ltd.

DOI: 10.1002/rcm