

How Does High Resolution ES-FTICR-MS Under Broadband Conditions Enhance Characterization of Proteins and Peptide Mixtures?

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The unambiguous characterization of important proteins such as hemoglobin requires analytical technology that can provide detailed identification at the molecular level. Mass spectrometry has shown remarkable promise for such studies, due to its ability to measure accurately biomolecules whose molecular masses are in the kilodalton region. In particular, electrospray Fourier transform ion cyclotron resonance mass spectrometry (ES-FTICR-MS) provides capabilities for high resolution, accurate mass measurement as well as ion manipulation techniques for probing ion structure. The objective of this report is to illustrate how high resolution ES-FTICR-MS can be conducted under broadband ion detection conditions for the identification and characterization of a variety of important proteins. To demonstrate this point, this report will be organized into three sections; 1) demonstration of the ORNL ES-FTICR-MS instrument with ubiquitin as a model protein, 2) examination of a variety of hemoglobin standards from different organisms to verify detection and identification of the alpha and beta chains, and finally 3) identification and characterization of hemoglobin variants from different mouse strains.

All mass spectra discussed in this report were acquired with a high resolution IonSpec (Irvine, CA) ES-FTICR-MS installed at ORNL. This system is equipped with a 7-Tesla actively-shielded superconducting magnet. The vacuum system is pumped with a split-turbomolecular pump and two cryopumps. The base pressure in the system is $< 1 \times 10^{-10}$ Torr; the operating pressure during electrospray acquisition is $< 3 \times 10^{-10}$ Torr. Ions generated by the high voltage potential between the electrospray needle and the metal end cap are transferred through a heated glass capillary and skimmer into a dc-rf hexapole. Ion accumulation is accomplished in the hexapole for a few hundred milliseconds, and a mechanical shutter is then temporarily opened (few milliseconds) to inject the ions into the quadrupole transfer system. The ions are finally trapped in an open-ended capacitively-coupled cylindrical analyzer cell. This mode of operation allows the low base pressure to be maintained in the analyzer cell. The ability to acquire transient signals up to 8 Mbyte and analyze with Fast Fourier Transform Processing provides broadband resolutions of about 200,000 (FWHM) at m/z 1000.

Initial instrument performance verification was conducted with ubiquitin as a model protein. ES-FTICR-MS of this biomolecule reveals multiply-charged ions ranging from $(M+7H)^{7+}$ at m/z 1225 to $(M+11H)^{11+}$ at m/z 780. An automated deconvolution routine assigns the charge states based on isotopic resolution. The deconvoluted molecular mass spectrum for ubiquitin revealed an isotopically-resolved molecule whose mass accuracy was within ten millimass units of the calculated value. For broadband signal acquisition under these experimental conditions, the transient lasted for at least 4 seconds, permitting a 2Mbyte time-domain signal to be acquired. This provided a broadband resolution of about 200,000 (FWHM) for the $(M+9H)^{9+}$ ion at m/z 953. Medium resolution ion isolation is accomplished with a conventional arbitrary waveform function generator; whereas high-resolution ion isolation ($> 15,000$ FWHM) can be attained with additional application of a rf burst excitation event. For example, this latter procedure provided a means of isolating a single isotopic peak of a single charge state for ubiquitin. Off-resonance collisional dissociation experiments are conducted by firing a pulse valve during the ion activation process to increase the collisional gas pressure. Pumpdown to pressures in the mid-low 10^{-10} Torr region is accomplished before ion detection.

In order to evaluate this ES-FTICR-MS for hemoglobin proteins, a series of hemoglobin standards from horse, bovine, and human sources were examined. For example, standard house hemoglobin (CAS 9047-

09-0) contains an alpha chain at Mr = 15114 and a beta chain at Mr = 16008 Da. An alpha chain variant at Mr = 15098 is also known and corresponds to a Y → F amino acid substitution at position 24. The ES-FTICR-MS of this horse hemoglobin protein sample (20 uM concentration in 50:50 water methanol) revealed the presence of several protein species. The deconvoluted molecular mass spectra verified the presence of the major alpha chain (15114 Da), the alpha variant (15098 Da), the beta chain (16008 Da), and an unknown beta chain variant (16104 Da). Likewise, examination of bovine, human, and human sickle cell hemoglobins yielded mass spectral confirmation of the expected alpha and beta chain proteins.

The major focus of this high-resolution ES-FTICR-MS research is to characterize mouse hemoglobin variants that provide information about single nucleotide polymorphisms. To this goal, two different mouse stains (C3H and B6) were examined. The hemoglobin of the North American Wild Mouse (*mus musculus*) consists of a 14954 Da alpha chain and a 15709 beta chain. The C3H mouse has an identical beta chain, but the C(1) allele for this species generates two alpha chain variants. The major alpha chain for the C3H mouse has a molecular mass of 15010 Da, and corresponds to a variant with two amino acid substitutions: G → V at position 25 and V → I at position 62. The minor alpha chain for the C3H mouse has a molecular mass of 14981 Da, and corresponds to a single amino acid change: S → N at position 68. The ES-MS of the C3H mouse hemoglobin indicates the presence of multiple proteins, as shown in Figure 1. The deconvoluted molecular mass spectra reveal the presence of both alpha chains (15010 and 14981), as shown in Figure 2, as well as the beta chain at 15709. Enzymatic digestion of the C3H mouse hemoglobin was conducted with trypsin to characterize the peptide sequence of the alpha and beta proteins. The deconvoluted spectra of this peptide sample revealed the characteristic fragments from both alpha chains along with the beta chain, confirming the molecular mass measurements. ES-MS of the B6 black mouse confirmed the expected alpha chain at 14981 Da and the beta chain at 15616 Da. By ejecting the major ions from the B6 mouse hemoglobin sample, two beta chain variants (15980 and 16535 Da) were detected in low abundance. Work is in progress to identify these biomolecules.

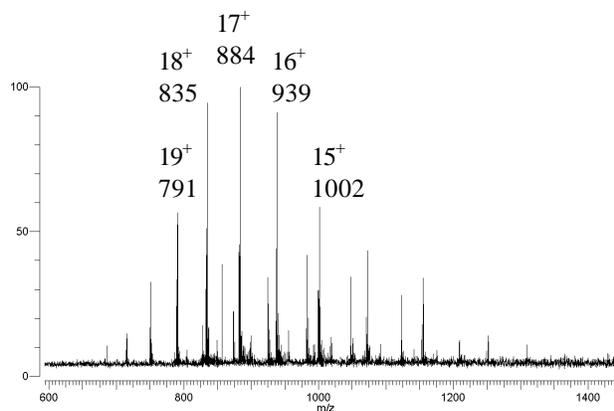


Figure 1.

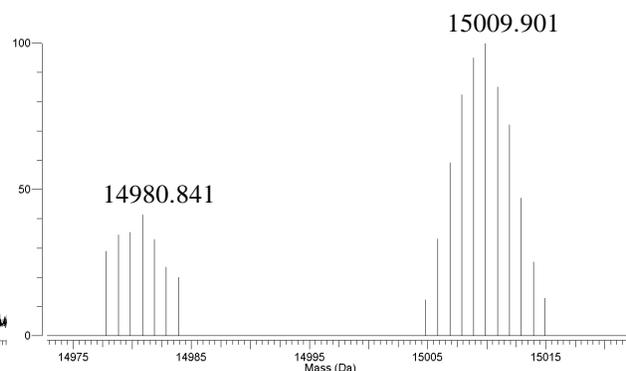


Figure 2.

In summary, high resolution ES-FTICR-MS shows tremendous potential for the identification and characterization of hemoglobin variants. This technique should provide important information for characterizing mouse hemoglobin mutations.

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