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Ion Mobility – Mass Spectrometry Techniques for Determining the Structure and Mechanisms of Metal Binding Oligopeptides

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TITLE:

Ion Mobility-Mass Spectrometry Techniques for Determining the Structure and Mechanisms of Metal Ion Recognition and Redox Activity of Metal Binding Oligopeptides

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ABSTRACT:

Ion mobility-mass spectrometry and molecular modeling techniques can characterize the selective metal chelating performance of designed metal-binding peptides and the copper-binding peptide methanobactin. Developing new classes of metal chelating peptides will help lead to therapeutics for diseases associated with metal ion misbalance.

SUMMARY:

Electrospray ionization (ESI) can transfer an aqueous-phase peptide or peptide complex to the gas-phase while conserving its mass, overall charge, metal-binding interactions, and conformational shape. Coupling ESI with ion mobility-mass spectrometry (IM-MS) provides an instrumental technique that allows for simultaneous measurement of a peptide's mass-to-charge (m/z) and collision cross section (CCS) that relate to its stoichiometry, protonation state, and conformational shape. The overall charge of a peptide complex is controlled by the protonation of 1) the peptide's acidic and basic sites and 2) the oxidation state of the metal ion(s). Therefore, the overall charge state of a complex is a function of the pH of the solution that affects the peptides metal ion binding affinity. For ESI-IM-MS analyses, peptide and metal ions solutions are prepared from aqueous-only solutions, with the pH adjusted with dilute aqueous acetic acid or ammonium hydroxide. This allows for pH dependence and metal ion selectivity to be determined for a specific peptide. Furthermore, the m/z and CCS of a peptide complex can be used with B3LYP/LanL2DZ molecular modeling to discern binding sites of the metal ion coordination and tertiary structure of the complex. The results show how ESI-IM-MS can characterize the selective chelating performance of a set of alternative metal-binding peptides and compare them to the copper-binding peptide methanobactin.

INTRODUCTION:

Copper and zinc ions are essential for living organisms and crucial to processes including oxidative protection, tissue growth, respiration, cholesterol, glucose metabolism, and genome reading[1]. To enable these functions, groups such as the thiolate of Cys, imidazole of His[2,3], (more rarely) thioether of methionine, and carboxylate of Glu and Asp selectively

incorporate metals as cofactors into the active sites of metalloenzymes. The similarity of these coordination groups raises an intriguing question regarding how the His and Cys ligands selectively incorporate either Cu(I/II) or Zn(II) to ensure correct functioning.

Selective binding is often accomplished by acquisition and trafficking peptides, which control Zn(II) or Cu(I/II) ion concentrations[4]. Cu(I/II) is highly reactive and causes oxidative damage or adventitious binding to enzymes, so its free concentration is tightly regulated by copper chaperones and copper-regulating proteins that transport it safely to various locations in the cell and tightly control its homeostasis[5,6]. Disruption of copper metabolism or homeostasis is directly implicated in Menkes and Wilson's disease[7] as well as cancers[7] and neural disorders, such as prion[8] and Alzheimer's disease[9].

Wilson's disease is associated with increased copper levels in the eyes, liver and sections of the brain, where the redox reactions of Cu(I/II) produces reactive oxygen species, causing hepatolenticular and neurological degeneration. Existing chelation therapies are the small thiol amino acid penicillamine and triethylenetetramine. Alternatively, the methanotrophic copper-acquisition peptides methanobactin (mb)[10,11] exhibit therapeutic potential because of their high binding affinity for Cu(I)[12]. When the methanobactin (mb-OB3b) from *Methylosinus* trichosporium OB3b was studied in an animal model of Wilson's disease, copper was efficiently removed from the liver and excreted through the bile[13]. In vitro experiments confirmed that mb-OB3b could chelate the copper from the copper metallothionein contained in the liver cytosol[13]. Laser ablation inductively coupled plasma mass spectrometry imaging techniques have investigated the spatial distribution of copper in Wilson's disease liver samples[14-16] and shown that mb-OB3b removes the copper with short treatment periods of only 8 days[17].

The mb-OB3b will also bind with other metal ions, including Ag(I), Au(III), Pb(II), Mn(II), Co(II), Fe(II), Ni(II), and Zn(II)[18,19]. Competition for the physiological Cu(I) binding site is exhibited by Ag(I) because it can displace Cu(I) from the mb-OB3b complex, with both Ag(I) and Ni(II) also showing irreversible binding to Mb which cannot be displaced by Cu(I)[19]. Recently, a series of alternative metal binding (amb) oligopeptides with the 2His-2Cys binding motif have been studied[20,21], and their Zn(II) and Cu(I/II) binding properties characterized. Their primary amino acid sequences are similar, and they all contain the 2His-2Cys motif, Pro and an acetylated N-terminus. They mainly differ from mb-OB3b because the 2His-2Cys motif replaces the two enethiol oxazolone binding sites of mb-OB3b.

Electrospray ionization coupled with ion mobility-mass spectrometry (ESI-IM-MS) provides for a powerful instrumental technique for determining the metal-binding properties of peptides because it measures their mass-to-charge (*m/z*) and collision cross section (CCS) while conserving their mass, charge, and conformational shape from the solution-phase. The *m/z* and CCS relate to the peptides stoichiometry, protonation state, and conformational shape. Stoichiometry is determined because the identity and number of each element present in the species is explicitly identified. The overall charge of the peptide complex relates to the protonation state of the acidic and basic sites and the oxidation state of the metal ion(s). The CCS gives information of the conformational shape of the peptide complex because it measures the rotational averaged size which relates to the tertiary structure of the complex. The overall charge state of the complex is also a function of pH and affects the

peptide's metal ion binding affinity because the deprotonated basic or acidic sites such as the carboxyl, His, Cys and Tyr are also the potential binding sites for the metal ion. For the analyses, the peptide and metal ion are prepared in aqueous solutions with the pH adjusted by dilute aqueous acetic acid or ammonium hydroxide. This allows for the pH dependence and metal ion selectivity to be determined for the peptide. Furthermore, the *m/z* and CCS determined by ESI-IM-MS can be used with B3LYP/LanL2DZ molecular modeling to discover the type of metal ion coordination and tertiary structure of the complex. The results shown in this article reveal how ESI-IM-MS can characterize the selective chelating performance of a set of amb peptides and compare them to the copper-binding peptide mb-OB3b.

PROTOCOL:

1. Preparation of reagents

1.1. Culture *Methylosinus trichosporium* OB3b, isolate the Cu(I)-free mb-OB3b[18,22,23], freeze-dry the sample and store at -80 °C until use.

1.2. Synthesize the amb peptides (>98% purity for amb_1, amb_2, amb_4; >70% purity for amb_7), freeze-dry the samples, and store them at -80 °C until use.

1.3. Purchase >98% purity manganese(II) chloride, cobalt(II) chloride, nickle(II) chloride, copper(II) chloride, copper(II) nitrate, silver(I) nitrate, zinc(II) chloride, iron(III) chloride and lead(II) chloride.

1.4. Purchase the poly-DL-alanine polymers used as calibrants for measuring the collision cross sections of the amb species and HPLC grade or higher ammonium hydroxide, glacial acetic acid, and acetonitrile.

2. Preparation of stock solution

2.1 Peptide stock solution

2.11 Weigh accurately, using at least three significant figures, the mass of 10.0–20.0 mg of the mb-OB3b or amb in a 1.7 mL plastic vial.

NOTE: The weighed mass should yield either 12.5 mM or 1.25 mM, depending on the solubility of the peptide, when 1.00 mL of deionized (DI) water is added.

2.12 Using a pipet, add 1.00 mL of deionized water (>17.8 MΩ cm) to the weighed peptide sample to yield either the 12.5 mM or 1.25 mM solution. Place cap securely and mix thoroughly with at least 20 inversions.

2.13 Using a micropipet dispense 50.0 µL aliquots from the peptide sample into individually labelled 1.5 mL vials and store them at -80 °C until use.

2.2 Metal ion stock solutions

2.21 Weigh accurately, using at least three significant figures, the mass of 10.0–30.0 mg of the metal chloride or silver nitrate in a 1.7 mL vial.

NOTE: The weighed mass should yield 125 mM when 1.00 mL of DI water is added.

2.22 Add the 1.00 mL of DI water to the weighed metal sample in the 1.7 mL vial to yield the 125 mM solution. Place cap securely and mix thoroughly with at least 20 inversions.

2.3 Ammonium hydroxide stock solutions: prepare a 1.0 M acetic acid solution by diluting 57 µL of the 99.5% acetic acid solution with DI water to a final volume of 1.00 mL. Prepare a 1.0 M ammonium hydroxide solution by diluting 90 µL of the 21% ammonium hydroxide solution with DI water to a final volume of 1.00 mL. Make two successive dilutions of each solution by taking 100 µL of the 1.0 M solutions to prepare 0.10 M and 0.010 M acetic acid and ammonium hydroxide solutions.

2.4 Poly-DL-alanine stock solution: prepare the poly-DL-alanine (PA) by weighing 1.0 mg of PA and dissolving in 1.0 mL of DI water to give 1,000 ppm. Mix thoroughly. Using a micropipet, dispense 50.0 µL aliquots, and place each into a 1.7 mL vial and store at -80 °C.

3. Electrospray-ion mobility-mass spectrometry analysis

3.1 Clean the ESI entrance tubing and needle capillary thoroughly with about 500 µL of 0.1 M glacial acetic acid, 0.1 M ammonium hydroxide, and finally DI water.

3.2 Thaw a 50.0 µL aliquot of the 1,000 ppm PA stock solution and dilute it with 450 µL of DI water to give a 100 ppm PA. Pipet 100.0 µL of this solution and dilute it to 1.00 mL with 500 µL of DI water and 500 µL of acetonitrile to give 10 ppm PA solution.

3.3 Collect the negative and positive ion IM-MS spectra of the 10 ppm PA solution for 10 min each using native ESI-IM-MS conditions as described in the discussion section.

3.4 Thaw a 50.0 µL aliquot of the 12.5 mM or 1.25 mM amb stock solution and make successive dilutions with DI water to give a final concentration of 0.125 mM amb. Mix thoroughly each dilution.

3.5 Pipet 100.0 µL of the 125 mM metal ion stock solution, place in a 1.7 mL vial and dilute to 1.00 mL with DI water to give 12.5 mM metal ion. Repeat with two more successive dilutions to give a final 0.125 mM metal ion concentration. Mix thoroughly each dilution.

3.6 Pipet 200.0 µL of the 0.125 mM amb into a 1.7 mL vial, dilute with 500 µL of DI water, and mix the solution thoroughly.

3.7 Adjust the pH of the sample to 3.0 by adding 50 µL of 1.0 M acetic acid solution.

3.8 Add 200.0 µL of the 0.125 mM metal ion to the pH-adjusted sample. Add DI water to yield a final volume of 1.00 mL of the sample, mix thoroughly, and allow the sample to equilibrate for 10 min at RT.

3.9 Using a blunt nose syringe take 500 µL of the sample and collect the negative and positive ion ES-IM-MS spectra for 5 min each. Use the remaining 500 µL of the sample to record its final pH using a calibrated micro pH electrode.

3.10 Repeat steps 3.6–3.9, while modifying step 3.7 to adjust the pH to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, or 10.0 by adding new volumes of the 0.010 M, 0.10 M, or 1.0 M acetic acid or ammonium hydroxide solutions.

3.11 Collect the negative and positive ion ESI-IM-MS spectra of the 10 ppm PA solution for 10 min each.

4. Preparation of the metal ion titration of amb samples

4.1 Follow the steps described in steps 3.1–3.5.

4.2 Pipet 200.0 µL of the 0.125 mM amb into a 1.7 mL vial, dilute with 500.0 µL of DI water and mix the solution thoroughly.

4.3 Adjust the pH of the sample to pH = 9.0 by adding 80 µL of the 0.010 M ammonium hydroxide solution.

4.4 Add 28 µL of the 0.125 mM metal ion solution to give 0.14 molar equivalents of the metal ion, add DI water to make the final volume of the sample 1.00 mL, mix thoroughly, and allow the sample to equilibrate for 10 min at RT.

4.5 Using a blunt nose syringe take 500 µL of the sample and collect the negative and positive ion ES-IM-MS spectra for 5 min each. Use the remaining 500 µL of the sample to record its final pH using a calibrated micro pH electrode.

4.6 Repeat steps 4.2–4.5, while modifying step 4.3 to add an appropriate volume of the 0.125 mM metal ion solution to give either 0.28, 0.42, 0.56, 0.70, 0.84, 0.98, 1.12, 1.26, or 1.40 molar equivalents.

4.7 Collect the negative and positive ion IM-MS spectra of the 10 ppm PA solution for 10 min each.

5. Analysis of ESI-IM-MS pH titration data

5.1 From the IM-MS spectra identify which charged species of ambs are present by matching them to their theoretical m/z isotope patterns.

5.1.1. Open MassLynx and click on **Chromatogram** to open the Chromatogram window.

5.1.2. Go to the **File** menu and **Open** to locate and open the IM-MS data file.

5.1.3. Extract the IM-MS spectrum by right-clicking and dragging across the chromatogram

and releasing. The spectrum window will open showing the IM-MS spectrum.

5.1.4. In the spectrum window, click on **Tools** and **Isotope model**. In the isotope modeling window, enter the molecular formula of the amb species, check the **Show charged ion** box, and enter the charge state. Click **OK**.

5.1.5. Repeat to identify all the species in the IM-MS spectrum and record their m/z isotope range.

5.2 For each amb species, separate any coincidental m/z species and extract their arrival time distributions (ATD) using their m/z isotope patterns to identify them.

5.2.1. In MassLynx click on **DriftScope** to open the program. In DriftScope click on **File** and **Open** to locate and open the IM-MS data file.

5.2.2. Use the mouse and left-click to zoom in on m/z isotope pattern of the amb species.

5.2.3. Use the **Selection tool** and left mouse button to select the isotope pattern. Click the **Accept current selection** button.

5.2.4. To separate any coincidental m/z species use the **Selection tool** and left mouse button to select the ATD time-aligned with isotope pattern of the amb species. Click the **Accept current selection** button.

5.2.5. To export the ATD, go to **File | Export to MassLynx**, then select **Retain Drift Time** and save file in appropriate folder.

5.3 Determine the centroid of the ATD and integrate the area under the ATD curve as a measure of the species population.

5.3.1. In the Chromatogram window of MassLynx open the saved exported file. Click on **Process | Integrate** from the menu. Use a peak-to-peak amplitude setting of 100 and click **OK**.

5.3.2. Record the centroid ATD (t_A) and the integrated area as shown on the **Chromatogram** window. Repeat for all saved amb and PA IM-MS data files.

5.4 Use the integrated ATD for all extracted amb species of either the positive or negative ions at each titration point to normalize to a relative percentage scale.

5.4.1. Enter the identities of the amb species and their integrated ATD at each pH into a spreadsheet.

5.4.2. For each pH, use the sum of the integrated ATDs to normalize the individual amb's ATD to a percentage scale.

5.4.3. Plot the percent intensities of each amb species vs. pH in a graph to show how the

population of each species varies as a function of pH.

6. Collision cross-sections

6.1 Using a spreadsheet, convert the CCSs (Ω) of PA negative[25,26] and positive[27] ions measured in He buffer gas[28] to corrected CCS (Ω_c) using **Equation 1** below, where: z = ion charge; e_c = electron charge (1.602×10^{-19} C); m_{N2} = mass of N_2 gas (Da); and m_{ion} = ion mass.[29]

$$\Omega_c = \frac{\Omega}{ze_c\sqrt{\left(\frac{1}{m_{N_2}} + \frac{1}{m_{ion}}\right)}} \quad (1)$$

6.2 Convert the average arrival times (t_A) of the PA calibrants and amb species into drift times (t_D) using **Equation 2** below, where: c = the enhanced duty cycle delay coefficient (1.41), and m/z is the mass-to-charge of the peptide ion.

$$t_D = t_A - \frac{c\sqrt{m/z}}{1000} \quad (2)$$

6.3 Plot the PA calibrants' t_D vs. their Ω_c. Then, using a least-squares regression fit of **Equation 3** shown below, determine the A' and B values, where: A' is the correction for the temperature, pressure, and electric field parameters; and B compensates for the nonlinear effect of the IM device.

$$\Omega_c = A' t_D^B \quad (3)$$

6.4 Using these A' and B values and the centroid t_D value from the ATD of the ambs determine their Ω_c using **Equation 3** and their Ω using **Equation 1**. This method provides CCSs for the peptide species with estimated absolute errors of about 2%[25-27].

7. Computational methods

7.1 Use the B3LYP/LanL2DZ level of theory, comprising of the Becke 3-parameter hybrid functionals[30] and the Dunning basis set[31] and electron core potentials[32,33,34] to locate geometry-optimized conformers for all possible types of coordinations of the observed *m/z* amb species[35].

NOTE: For details of how to build and submit calculations refer to the GaussView usage in **Supplementary File**.

7.2 Compare the predicted free energy of each of the conformers and calculate their theoretical CCSs using the ion-scaled Lennard-Jones (LJ) method from the Sigma program[36].

7.3 From the lowest free energy conformers determine which conformer exhibits the LJ CCS which agrees with the IM-MS measured CCS to identify the tertiary structure and type of coordination for the conformers observed in the experiment.

REPRESENTATIVE RESULTS:

Metal binding of amb_1

The IM-MS study[20] of amb_1 (**Figure 1A**) showed that both Cu(I/II) and Zn(II) bound to amb_1 in a pH-dependent manner (**Figure 2**). However, Cu(I/II) and Zn(II) bound to amb_1 through different reaction mechanisms at different coordination sites. For example, adding Cu(II) to amb_1 resulted in oxidation of amb_1 (amb_{1ox}) by disulfide bridge formation, and at a pH of >6, the $[amb_{1ox}-3H+Cu(II)]^-$ ion (**Figure 2A**) was formed. This indicated the deprotonation of two imidazoliums, carboxyl group, and two additional sites that were coordinating Cu(II).

Molecular modeling of the $[amb_{1ox}-3H+Cu(II)]^-$ ion using B3LYP/LanL2DZ determined the lowest energy complex was Cu(II) coordinated via the imidazole δN of His_1 and the deprotonated nitrogens of the backbone amide groups of Cys_2 and Gly_3. However, below a pH of 6, adding Cu(II) to amb_1 formed a m/z isotope pattern that could only be accounted for by Cu(I) binding, forming the $[amb_{1ox}+Cu(I)]^+$ ion (**Figure 2B**). In contrast, a pH higher than 6 caused the *m/z* isotope pattern to decrease 1 m/z, accounted for by the positive charged $[amb_{1ox}-H+Cu(II)]^+$ ion. Adding Zn(II) did not oxidize amb_1, and Zn(II) binding was observed at a pH of >6, primarily forming the $[amb_1-3H+Zn(II)]^-$ ion (**Figure 2C**). This indicated the deprotonation of the imidazoliums, thiol, and carboxyl groups. Molecular modeling of the $[amb_1-3H+Zn(II)]^-$ ion determined the lowest energy conformers to be either tetrahedral Zn(II) coordination via 2His-2Cys or His-2Cys and the carboxylate of the C-terminus.

Multiple Cu(I) binding of amb_2

The redox reactions between Cu(II) and amb_2 (**Figure 1B**) resulted in Cu(I) binding. This was studied in more detail using IM-MS, UV-Vis spectrophotometry, and B3LYP molecular modeling[37]. The main products of the Cu(II) titration of amb_2 at a pH of 5 were amb_2 oxidation (through disulfide bridge formation) and the unoxidized amb_2 species coordinating three Cu(I) ions.

A search using the B3LYP/LanL2DZ method located two low-energy complexes contending for the 3Cu(I) coordinated species. The first was the complex shown in **Figure 3A**, where the 3Cu(I) ions were coordinated via the bridging thiolate groups[38] of Cys_2 and Cys_6 (of His_1) as well as δN_1 and δN_5 (of His_5). The second complex (3c) has a salt bridge between the protonated His_1 side group and C-terminal carboxylate group. These results suggest that at a pH of 3.0–6.0, the principal amb_2+3Cu(I) complex is the salt-bridged structure, which can be successfully transferred from solution to gas-phase with only minimal structural rearrangement.

The theoretical LJ CCS of 209 ± 6 $Å^2$, calculated using the Sigma program[36] for complex 3c, agreed with the IM-MS measured CCS, indicating that 3c represents $[amb_2-2H+3Cu(I)]^+$ conformation at pH 3.0-6.0. However, at a pH of >6, this complex was not observed by IM-MS, probably because further deprotonation of His_1 (pK_a= 6.0) results in an overall neutral complex. Once the imidazoleum group of His_1 is deprotonated, 3Cu(I) coordination may convert to the bridging thiolate groups of Cys_2 and Cys_6 as well as δN_1 and δN_5 of His_1 and His_5, respectively (3a).

The pH dependence of amb_4 Cu(I/II)-binding and redox activity

The IM-MS and B3LYP techniques have been used to investigate the Cu(II) and pH titrations of amb_4 (**Figure 1C**) and identified monomer, dimer, trimer, and tetramer complexes of amb_4 containing up to three Cu(I) ions or two Cu(II) ions for each monomer subunit[39]. The complexes also contained various numbers of disulfide bridges, and these products were produced whether or not the Cu(II) reactions with amb_4 were conducted in anaerobic or aerobic aqueous solutions.

Using the IM-MS technique, it was shown that these individual species could be separated and quantified even if they had overlapping isotope patterns because of differences in their arrival times (**Figure 4**). The identification and quantification of these closely related species is a task that no other instrumental or analytical technique can achieve. These IM-MS studies provide considerable insight into the pH-dependent redox reactions and exactly identified the numbers of inter- or intra-molecular disulfide bridges, number of Cu(I) or Cu(II) ions, and number of deprotonation sites in each of the complexes (**Figure 5**).

Moreover, measuring the complexes CCS also allowed the determination of each of the individual species conformational size, which was used with an extensive B3LYP/LanL2DZ search to locate conformers with structures that agreed with both the correct molecular stoichiometry and CCS measured by IM-MS. Through this method, the Cu(I/II) coordination of the various complexes were identified. The reactions between Cu(II) and amb_4 included the formation of dimers, trimers, and tetramers coordinating either Cu(I) or Cu(II), depending on the pH of the solution.

For example, in solutions that were mildly acidic (pH = 3.0–6.0), they primarily bound Cu(I) ions and were unoxidized, while in solutions that were slightly basic (pH = 8.0–11.0), they primarily bound Cu(II) ions and were oxidized by all the Cys forming disulfide bonds (**Figure 6**). The B3LYP/LanL2DZ determined that the Cu(I) ions were linear and bridged by the thiolate and imidazole groups, while the Cu(II) ions were chelated via distorted T-shaped or square planar geometries by an imidazole as well as the deprotonated backbone nitrogens of amide groups.

IM-MS analysis of mb-OB3b

The IM-MS studies[19,40] of mb-OB3b (**Figure 1D**) showed that in the gas-phase, Cu(I)-free mb-OB3b exists as three negatively charged species: $[\text{mb-OB3b}-\text{H}]^{-}$, $[\text{mb-OB3b}-2\text{H}]^{2-}$, and $[\text{mb-OB3b}-3\text{H}]^{3-}$, consistent with expected solution-phase behavior. Individual metal ion titrations were performed[19] to determine the metal ion selectivity of mb-OB3b. **Figure 7** shows the results of the selected metal ion titrations and shows that the apparent binding selectivity of mb-OB3b can be categorized as three major groups: 1) Cu(I) and Ag(I); 2) Ni(II), Zn(II) and Co(II); and 3) Pb(II), Fe(II), and Mn(II). This order of binding selectivity was shown to be in general agreement with that found by fluorescence quenching experiments[19] and isothermal titration calorimetry[18].

Comparison of mb-OB3b and amb_7 metal binding selectivity

The apparent binding selectivity of mb-OB3b was compared to the binding selectivity of amb_7 at a pH of 7. The amb_7 was designed with the same amino acid sequence as mb-OB3b, but with the two enethiol oxazolone groups replaced with two His-Cys groups. The amb_7 (**Figure 1E**) has a single disulfide bond between Cys_6 and Cys_{12}. The results of the formation of

negative charged complexes (**Figure 8**) showed that amb_7 preferred binding selectivity for Ni(II) and Zn(II) (60%), followed by Co(II) and Pb(II) (40%). Furthermore, there was about 20% Cu(II) binding. There was either trace or no amb_7 binding of Ag(I), Mn(II), or Fe(II). This compared to mb-OB3b's preferred binding selectivity of over 90% for Cu(I) and Ag(I) binding.

FIGURE AND TABLE LEGENDS:

Figure 1: Primary structures of the alternative metal binding (amb) and methanobactin (mb-OB3b) peptides. (A) Acetyl-His_1-Cys_2-Gly_3-Pro_4-His_5-Cys_6 (amb_1); **(B)** acetyl-His_1-Cys_2-Tyr_3-Pro_4-His_5-Cys_6 (amb_2); **(C)** acetyl-His_1-Cys_2-Gly_3-Ser_4-Tyr_5-Pro_6-His_7-Cys_8-Ser_9 (amb_4); **(D)** 1-(N-[mercapto-(5-oxo-2-(3-methylbutanoyl)oxazol-(Z)-4-ylidene)methyl]-Gly_1–Ser_2–Cys_3–Tyr_4)-pyrrolidin-2-yl-(mercapto-[5-oxo-oxazol-(Z)-4-ylidene]methyl)–Ser_5–Cys_6–Met_7 (mb-OB3b); and **(E)** acetyl-Leu_1-His_2-Cys_3-Gly_4-Ser_5-Cys_6-Tyr_7-Pro_8-His_9-Cys_{10}-Ser_{11}-Cys_{12}-Met_{13} (amb_7). Shading shows the: 2His-2Cys or enethiol-oxazolone binding sites (●); proline or pyrrolidine hinges (●); acetyl or methylbutanol group N-terminus (●); and tyrosine, which can stabilize metal ion coordination via a second solvation shell π–cation interaction (●).

Figure 2: Mean relative intensities of the alternative methanobactin (amb_1) acetyl-His_1-Cys_2-Gly_3-Pro_4-His_5-Cys_6 and metal-bound complex (amb_1+*X*) (where *X* = Cu or Zn). Observations were made during negative and positive ion mass spectrometry analyses of 1:1 molar ratio solution of amb:XCl_2 over the pH range of 3.0–11.0. Error bars show standard deviations of the means of both the relative intensity and pH from three replicate pH titration experiments. The 1:1 molar solution of amb:$CuCl_2$ resulted in the oxidation of amb (amb_{ox}) with Cys_2 and Cys_6, forming a disulfide bridge. **(A)** Negative ion analysis of amb:$CuCl_2$ showing $[amb_{ox}]^-$ and $[amb_{ox}+Cu(II)]^-$. **(B)** Positive ion analysis of amb:$CuCl_2$ showing $[amb_{ox}]^+$ and $[amb_{ox}+Cu(I/II)]^+$; the oxidation state of Cu in the complex was pH-dependent, being Cu(I) below a pH of 8 and Cu(II) above a pH of 8. **(C)** Negative ion analysis of amb:$ZnCl_2$ showing $[amb]^{n-}$ and $[amb+Zn(II)]^{n-}$. **(D)** Positive ion analysis of amb:$ZnCl_2$ showing $[amb]^{n+}$ and $[amb+Zn(II)]^{n+}$. This figure has been adapted from a previous publication[20].

Figure 3: Proposed structures of $[amb_2+3Cu(I)]^+$ using lowest energy and geometry-optimized structures located from the B3LYP/LanL2DZ level of theory. (**A**) 3 Cu(I) coordination via $\delta N_1\delta N_5$ of His_1 and His_5 and thiolate bridging thiolate groups of Cys_2 and Cys_6 with a theoretical cross-section of 217 ± 6 $Å^2$. (**B**) Illustration of the $\delta N_1\delta N_5$ and thiolate bridging coordination. **(C**) Salt bridged structure showing the 3 Cu(I) coordination via carboxylate terminal (Cys_6), δN_5, and thiolate bridging with a theoretical cross section of 209 ± 6 $Å^2$. **(D**) Illustration of the carboxylate terminal, δN_5, and thiolate bridging coordination. Bonding distances A, B, C, D, E, and F are shown in the unit of Å. This figure has been adapted from a previous publication[37].

Figure 4: IM-MS analysis of products of the 1:1 mixture of amb_4:Cu(II) at pH = 4.4. (A) Extracted isotope patterns for the $[amb_4-2H+3Cu(I)]^+$, $[diamb_4-4H+6Cu(I)]^{2+}$, $[triamb_4-6H+9Cu(I)]^{3+}$ and $[tetraamb_4-8H+12Cu(I)]^{4+}$ species. **(B)** Integration of the extracted arrival times of $[amb_4-2H+3Cu(I)]^+$, $[diamb_4-4H+6Cu(I)]^{2+}$, $[triamb_4-6H+9Cu(I)]^{3+}$ and $[tetraamb_4-8H+12Cu(I)]^{4+}$ were used to calculate their relative intensities. To calculate the percent relative intensities, the summation of the integrated area for all extracted species for each titration point was used to normalize to the percent scale.

Figure 5: Changing isotope pattern for singly Cu(I/II) bound amb_4 observed during the pH titration of molar equivalents of Cu(II):amb_4 at pH = 4.04, 6.02, and 9.98. At pH = 4.04, the experimental result primarily matches the isotope model for $[amb_4+Cu(I)]^+$. At pH = 6.02, there is a shift of -2 m/z, signifying the formation of the disulfide bridge (shown as oxidation of amb_{4ox}) and agreement with the isotope pattern for $[amb_{4ox}+Cu(I)]^+$. At pH = 9.98, there is a further shift of -1 m/z, signifying Cu(II) binding and the removal of a proton to maintain the +1 charge state, which then matches the isotope pattern for $[amb_{4ox}-H+Cu(II)]^+$.

Figure 6: Changing relative intensities of identities of the Cu(I/II) complexes of the monomer, dimer, and trimer of amb_4 over pH range of 3.0–11.0. (A) Monomer with one Cu(I/II) ion, **(B)** dimer with 2 Cu(I/II) ions, and **(C)** trimer with 3 Cu(I/II) ions. The captions note how many disulfide bonds were present in the complex. This figure has been adapted from a previous publication[39].

Figure 7: Percentage of formation of the Cu(I), Ag(I), Zn(II), Ni(II), Co(II), Mn(II), Pb(II), or Fe(II) complexes of methanobactin. Observed during the individual metal ion titrations of methanobactin. It should be noted that Cu(I) binding resulted from the addition of Cu(II) and Fe(II) binding from the addition of Fe(III). This figure has been adapted from a previous publication[19].

Figure 8: Comparison of the percentage of Cu(I/II), Ag(I), Zn(II), Ni(II), Co(II), Mn(II), Pb(II), or Fe(II) chelation by mb-OB3b and amb_7 at pH = 7. The comparison is for the formation of negatively charged ions.

Supplementary File. GaussView usage.

DISCUSSION:

Critical steps: conserving solution-phase behaviors for examination via ESI-IM-MS

Native ESI instrumental settings must be used that conserve the peptides stoichiometry, charge state, and conformational structure. For native conditions, the conditions in the ESI source such as the cone voltages, temperatures, and gas flows have to be optimized. Also, the pressures and voltages in the source, trap, ion mobility, and transfer traveling waves (especially the DC trap bias that controls injection voltage into the IM cell) must be checked for their influences on charge-state and ion mobility distributions.

The following are the typical operating conditions that were used in this work. The aqueous peptide samples were injected using a blunt nose 1.0 mL syringe using a 10 µL min^{-1} flow rate, 2.0 kV capillary voltage for positive ions (+) or −1.8 kV for negative ions (-), 130 °C source temperature, 250 °C desolvation, 20 V sampling cone, and 4.0 V extraction cone. The IM section was operated with 6.0 V entrance voltage to the trap cell with an argon pressure of 2.25 x 10^{-2} mbar using a 1.5 mL/min flow rate. The voltage for injecting ions (trap DC bias) into the IM cell was set at 12 V to avoid dissociation of ions as they initially collided with the nitrogen buffer gas. The IM cell separated ions based on their charge and collision cross-section and utilized a 0.52 mbar nitrogen pressure and 20.0 mL min^{-1} flow rate. The IM was operated with ramped 12.0–20.0 V (+) or 8.0–30.0 V (–) travelling wave heights and ramped

800–1,500 m s^{-1} (+) or 250–1,000 m s^{-1} (–) velocities for every sweep through the cell of the IM travelling wave. The transfer cell was operated with the same argon pressure as the trap cell and guided the IM resolved ions to the orthogonal time-of-flight mass-to-charge analyzer. The ion mobility-mass spectra were acquired by synchronizing the gated release of ions into the IM cell with the time-of-flight mass-to-charge analyzer.

Using native ESI conditions, solution-phase properties such as the charge state and conformational state are conserved during the IM-MS analyses. For example, the charge states of mb-OB3b and ambs observed during IM-MS analyses[20,37] were closely related to the charge states expected in the solution phase[40]. The mb-OB3b peptide is tetraprotic and forms only negatively charged ions during IM-MS analysis[40], whether Cu(I)-bound or Cu(I)-free, because it contains the C-terminus (p*K*a < 1.7), two enethiol oxazolone groups (p*K*a = 5.0 and 9.7), and Tyr group (p*K*a = 11.0)[42]. The ambs in their fully protonated form will have an overall charge of +2 because of the C-terminus (p*K*a ≈ 2), two His (p*K*a = 6.0), two Cys (p*K*a = 8.3), and Tyr (p*K*a = 11.0) sites[19,41]. Thus, they generally form positively charged ions at a pH of <6 and negatively charged ions at a pH of >6.

The ambs also showed clear pH-dependent Cu(I/II) binding behavior and redox activity in which Cu(I)-binding at a low pH transitioned to Cu(II) binding at a higher pH. The Cu(I/II) reactions included forming the oxidized amb species (amb_{ox}) that contained disulfide bonds and various multimers and multiple Cu(I/II) binding (**Figure 5,6**). These redox reactions are time-dependent and it was shown that the longer the time interval (up to 210 min) between sample preparation and IM-MS analyses the more oxidized products were observed[37]. Therefore, careful consideration of reaction time dependence on the observation of products is also required.

Limitations: IM-MS and theoretical collision cross-sections identify which type of coordination each metal ion prefers

To help interpret the IM-MS m/z and CCS data, an extensive search was conducted using the B3LYP/LanL2DZ level of theory. Geometry-optimized conformers with different coordination sites were compared between their predicted free energy and agreement with the CCS measured by IM-MS. Molecular modeling of these peptides and their complexes is limited by the type of electronic structure calculations that can be applied to these relatively large systems. Other methods that have been studied or recommended include work by Truhlar et al.[43], who found that M05-2X was the best DFT functional and PM7 and MNDO/d were good NDDO semi-empirical methods for Zn(II)-containing compounds[44]. These peptides have a large conformational space and thorough investigation to locate the lowest energy conformers must include comparing the various metal chelating sites, various cis- and trans-peptide bonds, salt-bridges, hydrogen bonding, and π-cation interaction between the aromatic Tyr side group and metal cation.

Significance with respect to existing methods: Cu(I/II) and other selected metal ion binding compared between mb-OB3b and ambs

X-ray crystallography and NMR spectroscopy are the most common techniques used for determining the atomic resolution of peptides tertiary structure. However, X-ray crystallography studies of metallopeptides are scarce due to problems with the crystallization of these complexes[45]. NMR is also not suitable for the interpretation of a sample where

562 closely related individual oligopeptide species are present[46]. Therefore, IM-MS and molecular
563 modelling are alternative techniques for studying peptide complexes especially those that
564 result from complex redox and Cu(I/II)-binding reactions[20,37,40,47]. The strength of IM-MS is
565 that it can resolve each of the products and identify their molecular identity by simultaneously
566 measuring their m/z and arrival times that relate to the stoichiometry, protonation state, and
567 conformational structure.
568
569 For example, the mb-OB3b will chelate a variety of metal ions, and its selectivity towards each
570 ion was displayed by the IM-MS metal ion titrations (**Figure 7**). The results showed the mb-
571 OB3b preference for binding Cu(I) and Ag(I), while comparing the results at a pH of 7 with
572 amb_7. **Figure 8** shows amb_7 preferentially binding Zn(II) and Ni(II). In general, the amb studies
573 showed that replacing the two enethiol-oxazolones with 2His-2Cys did not exclude Cu(I/II)-
574 binding, but it resulted in multiple Cu(I)-binding via linear-bridging coordination (**Figure 3**) as
575 opposed to the mononuclear Cu(I) binding of mb-OB3b's tetrahedral coordination[48]. Cu(II)
576 reduction was also mediated by thiol oxidation and disulfide bridge formation in contrast to
577 the existing disulfide bridge in apo-mb-OB3b and the high reduction potential for copper-
578 loaded mb-OB3b, which supports the strong preference for Cu(I)[49].
579
580 **Future applications**
581 Further IM-MS studies of amb peptides are underway, in which their primary sequence is
582 modified by replacing the His or Cys with Gly or Asp, while the Tyr residue is replaced with
583 either Gly or Phe. These studies are also being conducted in 10.0 mM ammonium acetate,
584 with the pH modified with ammonium hydroxide (for pH = 7, 8, and 9) to keep the total ionic
585 strength constant for each sample. These results will be published shortly.
586
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594
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596 The authors have nothing to disclose.
597
598 **REFERENCES:**
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Figure

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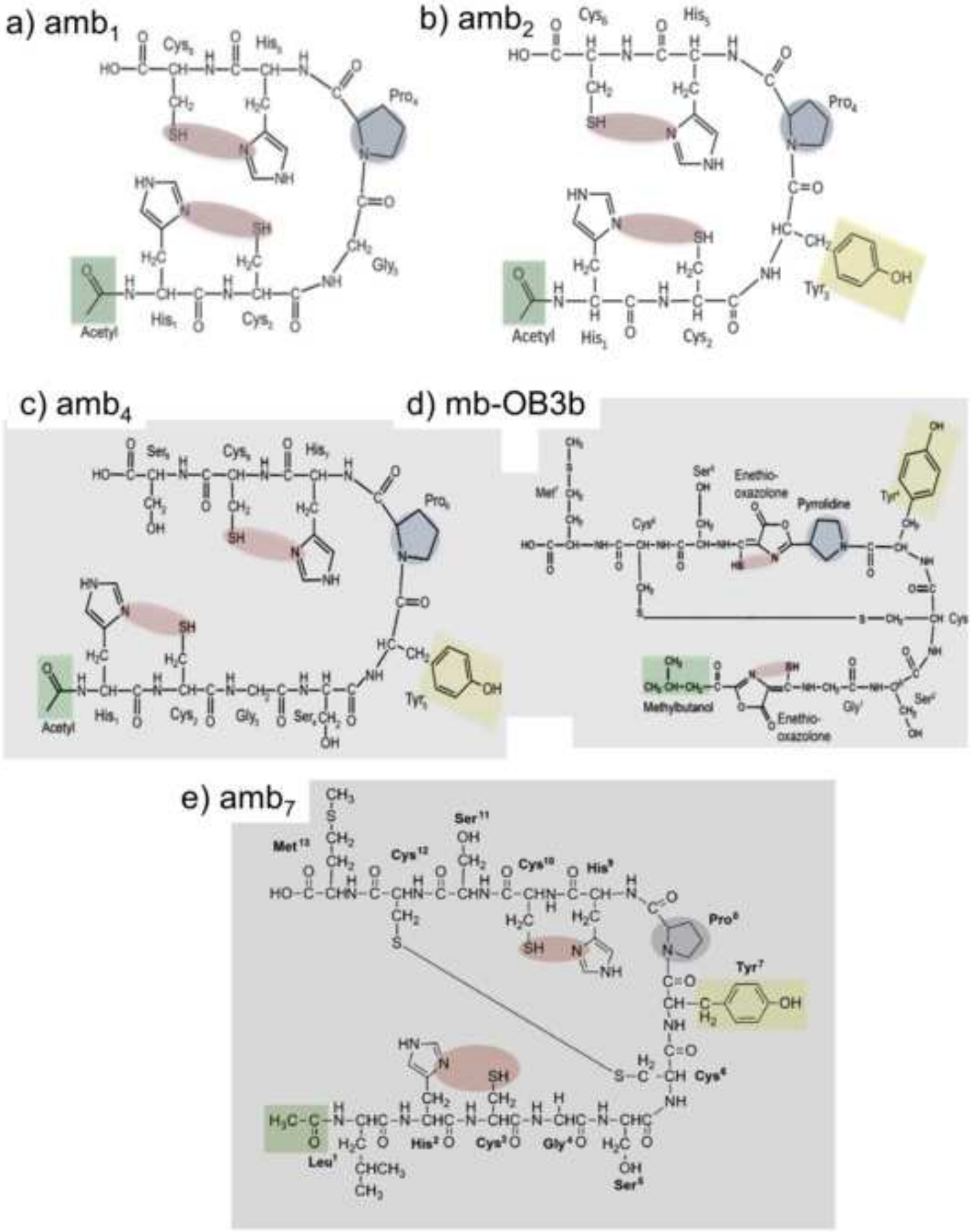

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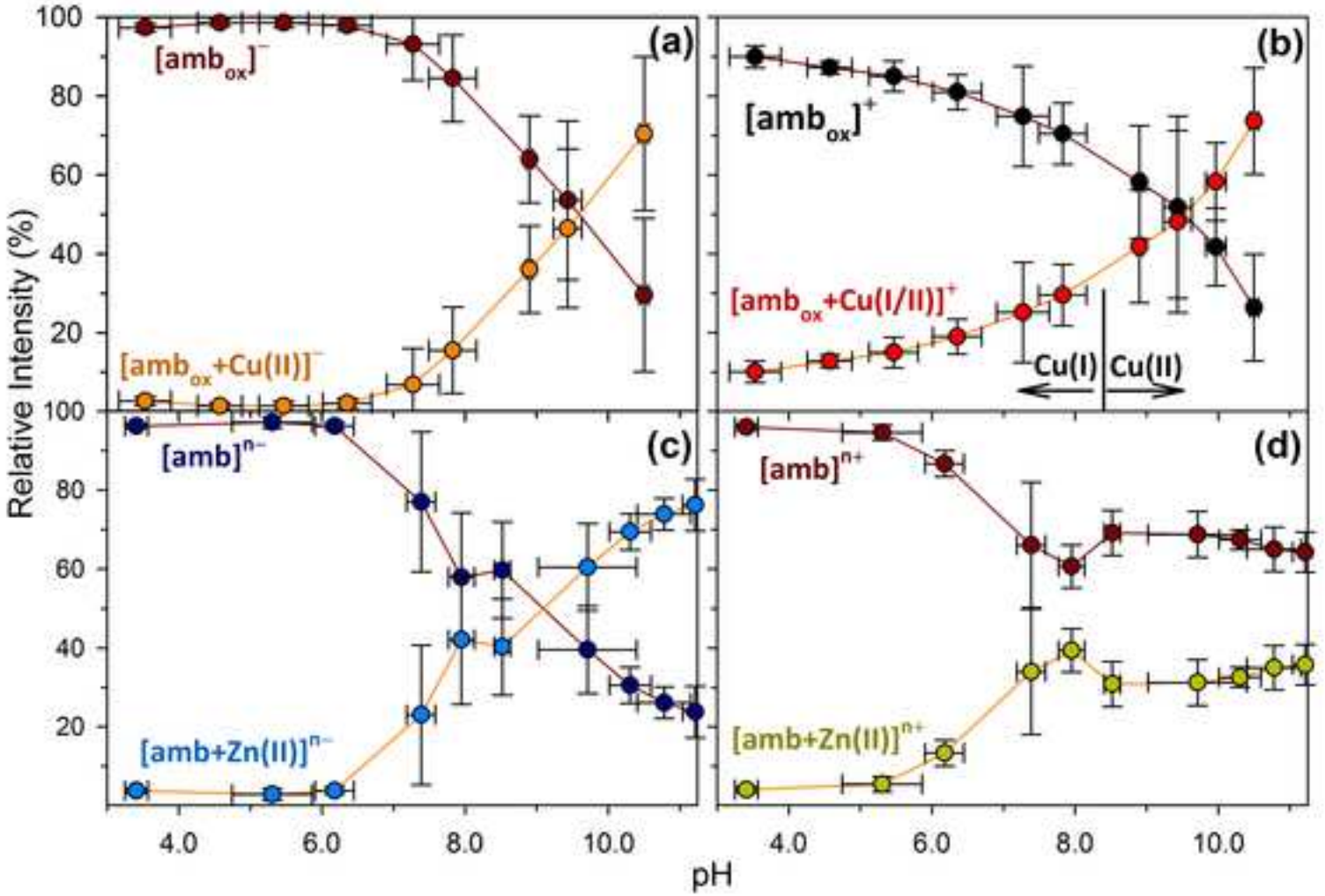
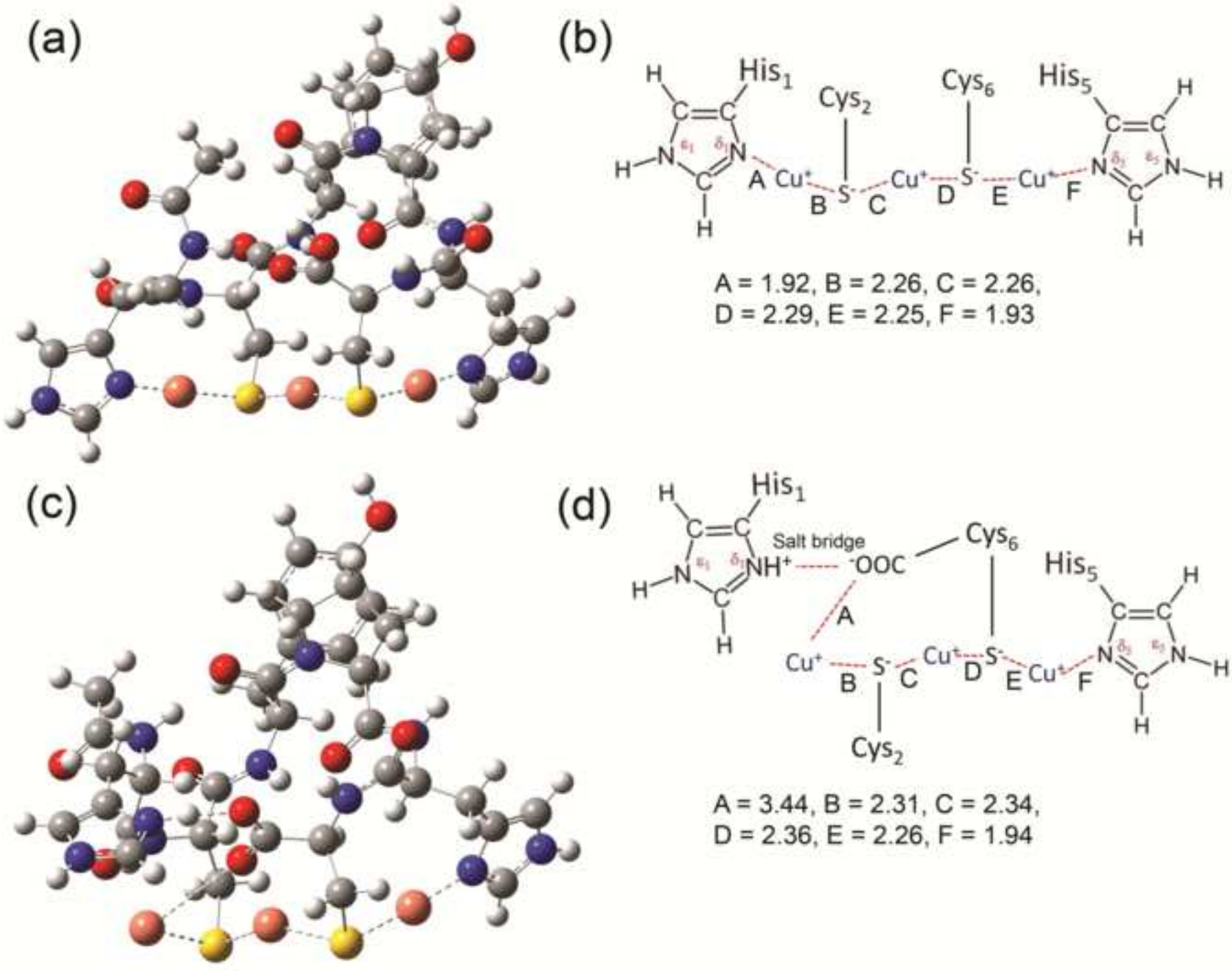


Figure



Figure

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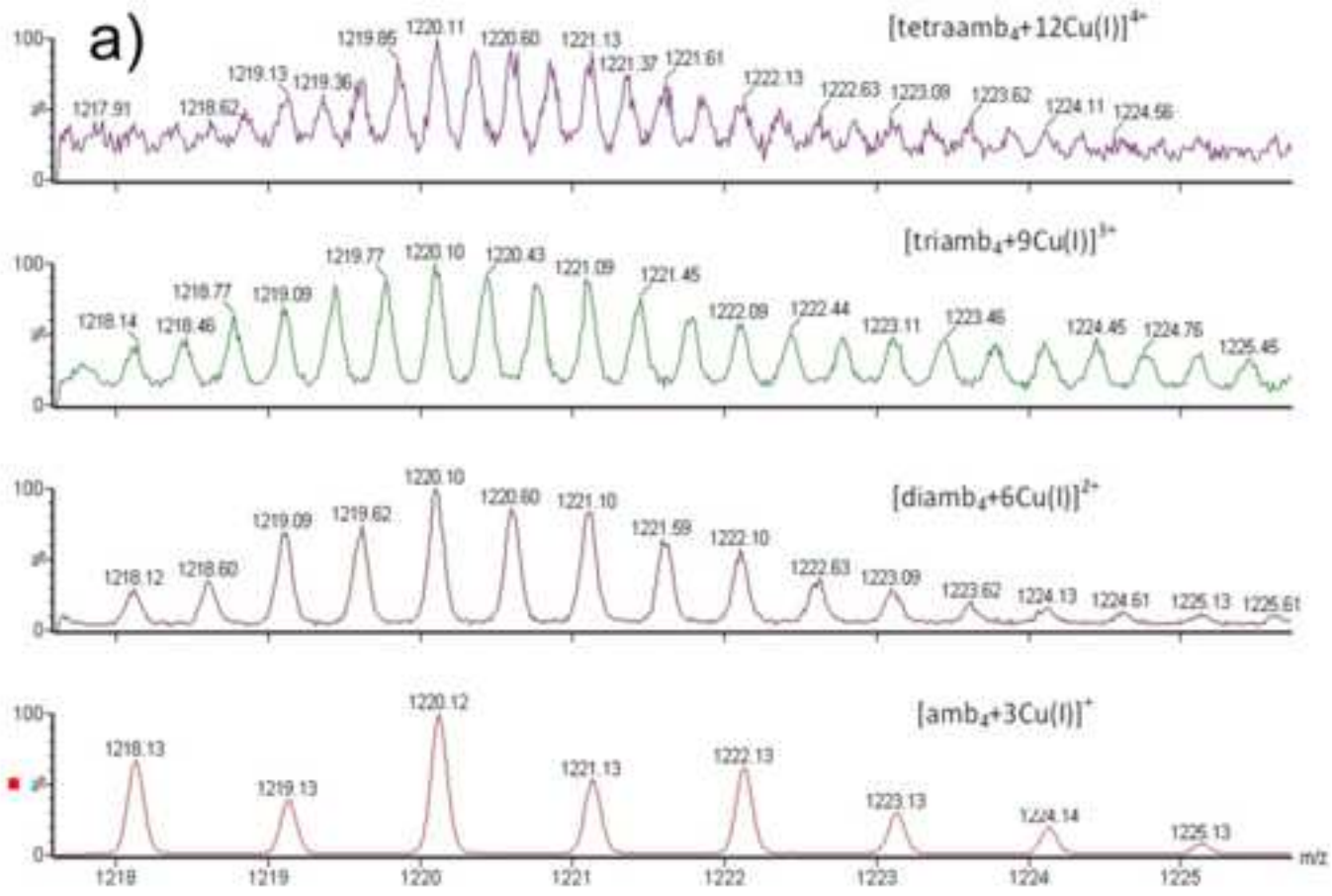

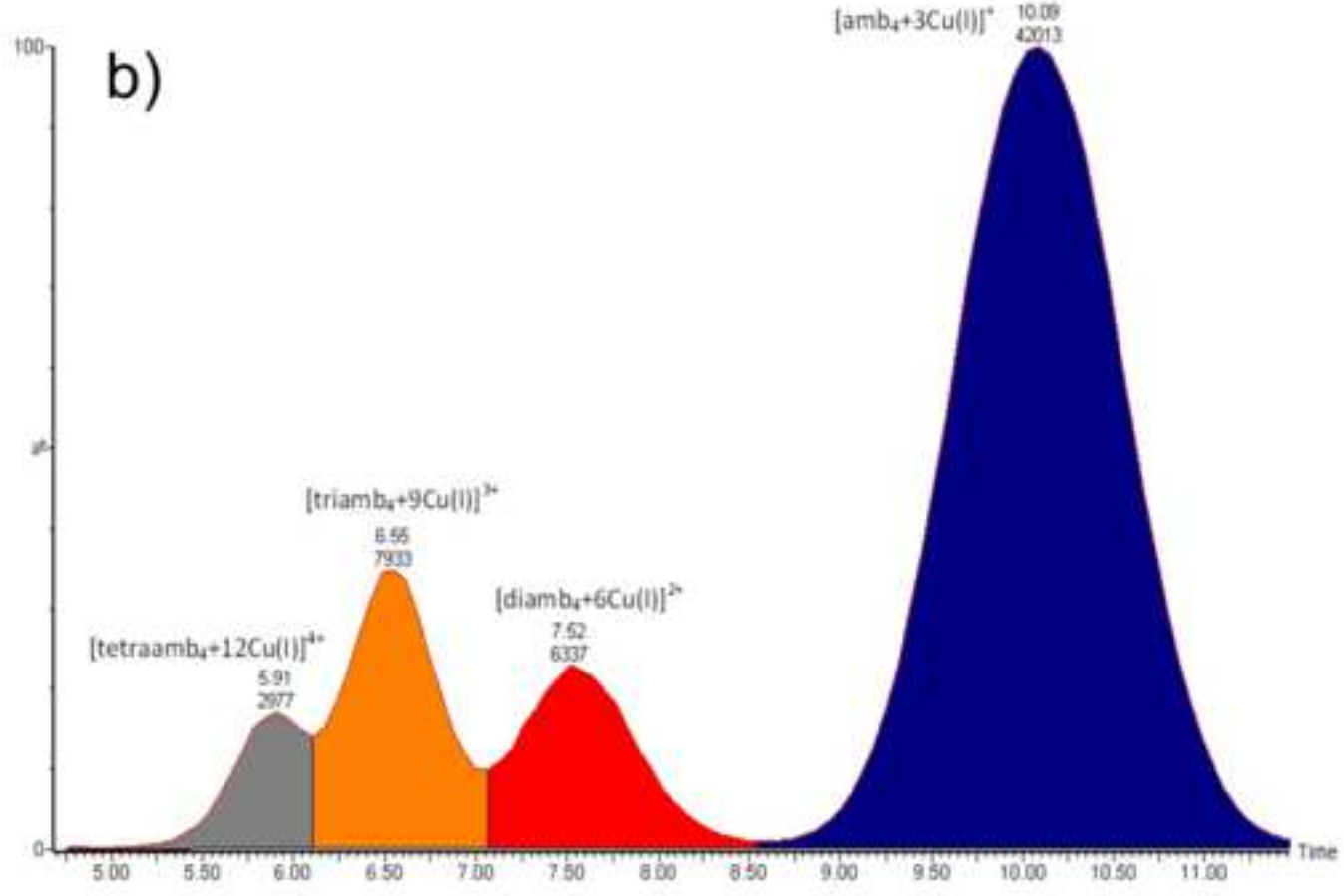

Figure

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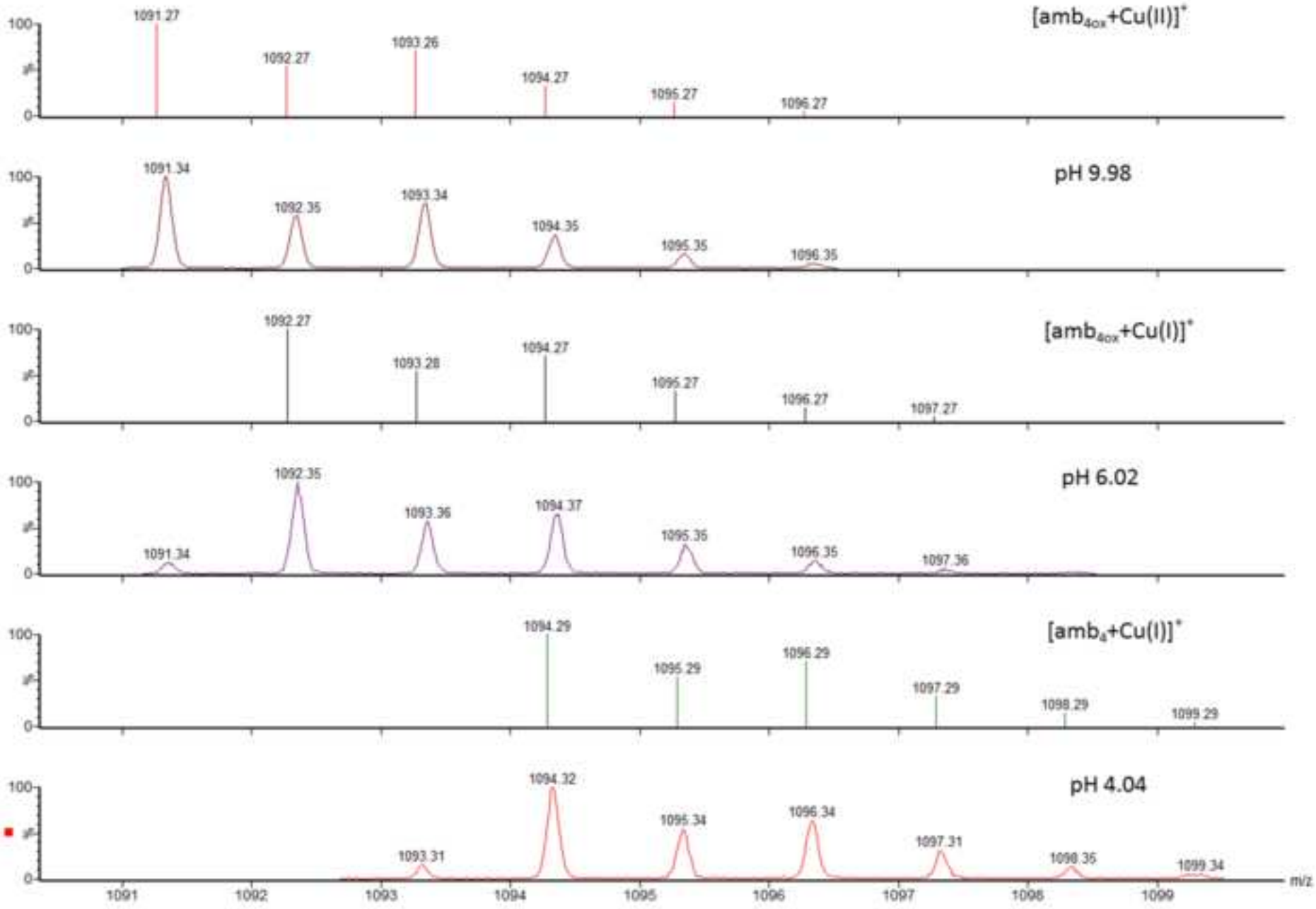

Figure

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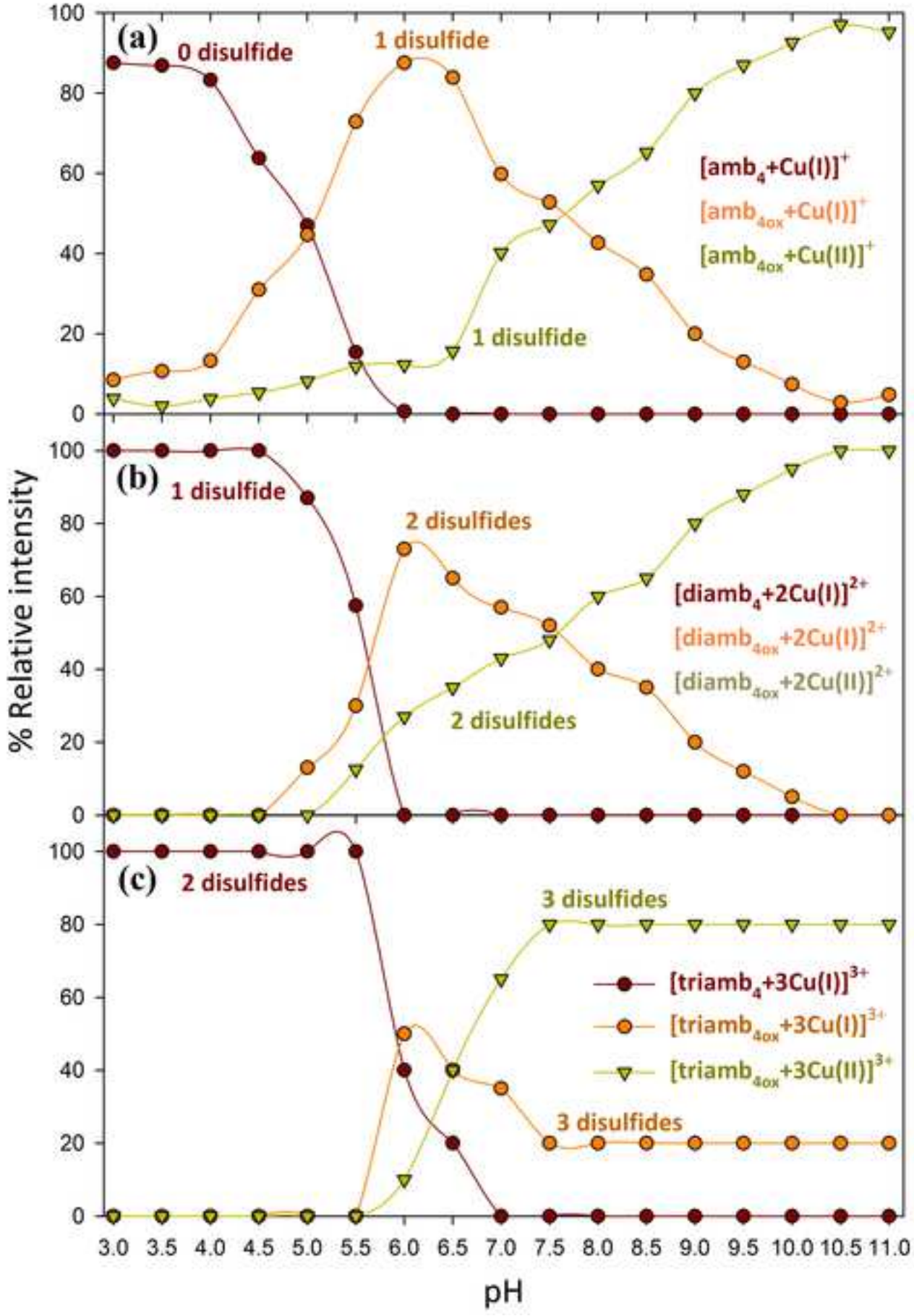

Figure

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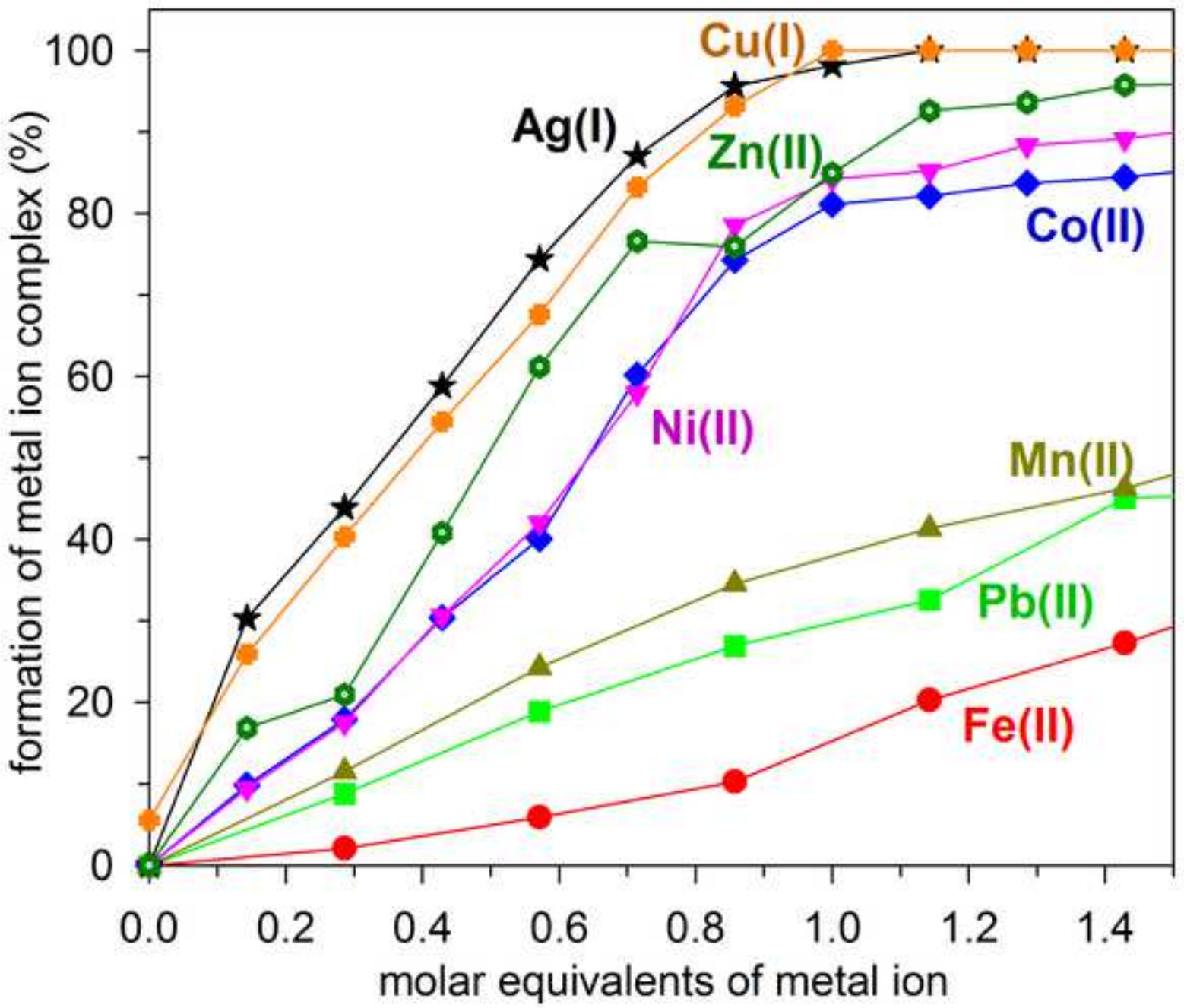

Figure

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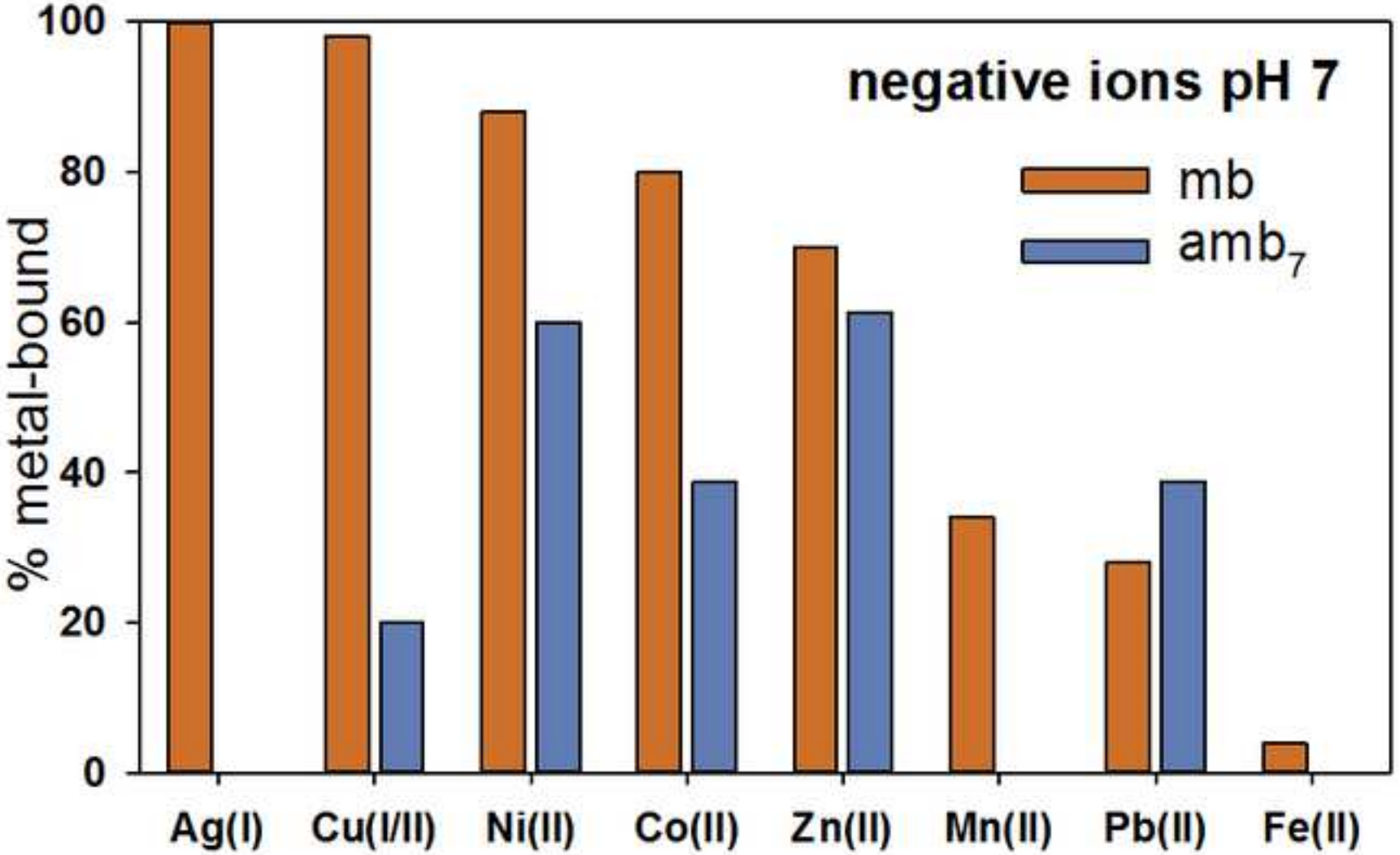

Name of Material/Equipment	Company	Catalog Number
acetonitrile HPLC-grade	Fisher Scientific (www.Fishersci.com)	A998SK-4
ammonium hydroxide (trace metal grade)	Fisher Scientific (www.Fishersci.com)	A512-P500
cobalt(II) chloride hexahydrate 99.99%	Sigma-Aldrich (www.sigmaaldrich.com)	255599-5G
copper(II) chloride 99.999%	Sigma-Aldrich (www.sigmaaldrich.com)	203149-10G
copper(II) nitrate hydrate 99.99%	Sigma-Aldrich (www.sigmaaldrich.com)	229636-5G
designed amb1,2,3,4,5,6,7 peptides	Neo BioLab (neobiolab.com)	
designed amb5B,C,D,E,F peptides	PepmicCo (www.pepmic.com)	
Driftscope 2.1 software program	Waters (www.waters.com)	
Freeze-dried, purified, Cu(I)-free mb-OB3b		
glacial acetic acid (Optima grade)	Fisher Scientific (www.Fishersci.com)	A465-250
Iron(III) Chloride Anhydrous 98%+	Alfa Aesar (www.alfa.com)	12357-09
lead(II) nitrate ACS grade	Avantor (www.avantormaterials.com)	128545-50G
manganese(II) chloride tetrahydrate 99.99%	Sigma-Aldrich (www.sigmaaldrich.com)	203734-5G
MassLynx 4.1	Waters (www.waters.com)	
nickel chloride hexahydrate 99.99%	Sigma-Aldrich (www.sigmaaldrich.com)	203866-5G
poly-DL-alanine	Sigma-Aldrich (www.sigmaaldrich.com)	P9003-25MG
silver nitrate 99.9%+	Alfa Aesar (www.alfa.com)	11414-06
Waters Synapt G1 HDMS	Waters (www.waters.com)	
zinc chloride anhydrous	Alfa Aesar (www.alfa.com)	A16281

Comments/Description
designed peptides were synthized by order
designed peptides were synthized by order
software analysis program
cultured and isolated in the lab of Dr. DongWon Choi (Biology Department, Texas A&M-Commerce)
software analysis program
quadrupole - ion mobility- time-of-flight mass spectrometer

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Author(s): E.N. Yousef, R. Sesham, J.W. McCabe, Rajpal Vungula, L.A. Angel

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CORRESPONDING AUTHOR

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Title:	ASSOCIATE PROFESSOR
Signature:	Laurence Angel
Date:	4/5/2019

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TEXAS A&M UNIVERSITY
COMMERCE

June 12th, 2019

Dear Bing Wu, Ph.D. Review Editor,

We have edited our article and would like to re-submit "Ion Mobility – Mass Spectrometry Techniques for Determining the Structure and Mechanisms of Metal Ion Recognition and Redox Activity of Metal Binding Oligopeptides" for consideration for publication in *JoVE*. The article illustrates our experimental methods of using traveling wave ion mobility – mass spectrometry (TWIMS) and density functional theory (DFT) to determine how effectively a series of modified 2His-2Cys motif oligopeptides can chelate Zn(II) and Cu(I/II) ions and compares them to the methanobactin peptide synthesized by methanotrophic bacteria. The paper should be of interest to wide range of readers interested in ion mobility - mass spectrometry techniques as applied to biochemical systems and in particular to using the TWIMS and DFT techniques for elucidating conformations and reactivity of peptides and metal ions that are important in many areas of biochemical research.

We have addressed the editors and reviewers comments below and our responses are shown in red.

Yours sincerely,

Dr. Laurence Angel
Associate Professor of Chemistry
Chemistry Department
Texas A&M University – Commerce

Tel. 903 886 5391

Co-authors: Enas N. Yousef, Ramakrishna Sesham, Jacob W. McCabe, and Rajpal Vangala.

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3. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please rewrite lines 60-63, 65-69, 281-291, 324-328. These sections have been rewritten and significant changes have been made.
4. The short abstract is over the 50 word limit. The short abstract has been modified to the 50 word limit.
5. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. The "Reagents and stock solutions" section has been modified.
6. Please add a one-line space between each of your protocol steps. added
7. Please avoid long step (more than 4 lines). modified
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9. Step 4.4: Please write this step in the imperative tense. modified
10. 5.2: Please write this step in the imperative tense. modified
11. Step 6: Please split this step into more substeps and ensure each step is written in the imperative tense. modified
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14. Step 9: Please split this step into more substeps and ensure each step is written in the imperative tense. modified
15. For each figure, please provide a title and a short description in Figure Legend. modified
16. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol Added
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique Added
d) The significance with respect to existing methods Added
e) Any future applications of the technique included
17. Please do not abbreviate journal titles for references. These are now corrected.

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18. The highlighted protocol steps are over the 2.75 page limit (including headings and spacing). Please highlight fewer steps for filming. The protocol section has been modified to fit in the 2.75 page limit.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors have submitted the results of metal complexes of methanobactin with comparison of alternative metal binding peptides. The used technique was the electrospray ionization - ion mobility-mass spectrometry, which was completed with DFT calculations. From the IM-MS data, however, much more conclusion were drawn than these experiments actually support.

To sum up, this manuscript would like to demonstrate how many type of information can be obtained from the IM-MS measurements, but without the primer experimental data and the reinforcement of the conclusions by using other methods is missing. I cannot accept these conclusions. Two new figures with primary data have been included to show how the combination of IM-MS and DFT can determine these conclusions.
So I suggest the rejection of this manuscript.

Major Concerns:
1) The authors have suggested the coordination mode of the Zn(II) complexes on the basis of the change of intensity of the species with different charge in the function of pH. The charge of the ligands, however, is changed also parallel with increasing of pH due to the deprotonation of thiol, carboxyl, imidazolium etc. groups and these processes were not taken into account. The deprotonation is taken into account and is critical to our evaluation. This is one of the strengths of this method as it measures mass-to-charge ratios and explicitly gives information on the deprotonation state and oxidation state of the metal ion of the peptide complex as a function of pH. One of the most important complexes is the singly-negative charged complex. As shown in Figures 2a and 2c this single charged complex increases above pH 6. For the complex to obtain this overall charge the thiol, carboxyl, imidazolium groups must be deprotonated, indicating their involvement with the coordination of the metal(II) at pH > 6. A new abstract and sections have been included in the introduction to make this clearer. Also, this is stated in various parts of the results and discussion in the paper.
2) On the other hand the presence of numerous side chain donor groups in the molecules could result in formation of different coordination isomers (with the same stoichiometry). The suggested structures of the complexes are only assumptions, but the IM-MS parameters do not give any evidence for the structure of the complexes. The suggested structures are based on locating the lowest free energy conformers predicted by the B3LYP method with the exact charge state (protonation state of the peptide and charge state of the metal ion) and mass (stoichiometry). The collision cross sections (rotationally averaged area of the complex in angstroms squared) of these B3LYP structures are determined using the ion-scaled Lennard-Jones (LJ) method which gives accurate measurements for these peptides. These CCS are

compared with the CCS experimentally measured by IM-MS of the molecule. Agreement between the CCS of the B3LYP structure and IM-MS CCS (along with the correct mass-to-charge) is used to support the proposed structures. The B3LYP modeling requires extensive time to locate a wide range of possible conformers with coordinations by the various ligands (you use your knowledge of the possible binding sites and compare them all) and is a very time consuming process. A table showing comparison of the single-negative ions of amb_5 is included to show this. The experimental protocol has been modified and the results and discussion to make this method clearer.

3) The manuscript does not demonstrate any primer experimental data (for example the stoichiometry and measured molecular mass data should be collected in Tables, etc.). Two new figures (Figures 4 and 5) showing primer experimental data and a table (Table 1) have now been included in the manuscript.

4) The figures represents only some example for the calculated data, but the conclusions were drawn for all six ligands and eight metal ions. These conclusions are based only on the data of one technique, so these are not acceptable. The goal of this paper is to show how IM-MS with B3LYP modeling can be used successfully to gain the type of information shown here because IM-MS gives the exact charge state, mass, and size of each individual resolved species. This type of information cannot be gained by any other technique! These results ***are*** supported by spectroscopic studies (UV-Vis and fluorescence quenching) and the papers where these techniques were included with the IM-MS and B3LYP analysis have been referenced. However, these spectroscopic methods can only give information of the whole ensemble of products produced from these reactions and not the individual products as IM-MS can by separating them all using their differences in m/z and their drift times in the ion mobility cell. These strengths of the IM-MS techniques have been stated more concisely and effectively within the script.

5) I cannot accept the answer for 3. and 4. questions, these answers are only assumptions, they are not proven facts. The answers to questions 3 and 4 are proven by the agreement between theory and experiment. As stated above the IM-MS analyses give the exact charge state (protonation state and charge state of the metal ion), mass (stoichiometry), and size (collision cross section) of *each* of the individually resolved species. These are then matched to the lowest energy B3LYP located molecules that exhibited the same mass-to-charge and shape/size as measured by IM-MS. The B3LYP modeling requires extensive time to locate all the possible conformers that could be formed by the coordinations by the various ligands (you use your knowledge of the possible binding sites) and is a time consuming process. The text has been modified with these details at various points to make it clearer how these final answers are supported by experiment and theory.

Minor Concerns:

6) Figure 5 shows the relative intensity of complexes of six ligands (A, B, C, … F). The connecting of these points is pointless. This figure has been deleted to make way for the two new figures that show primer experimental data.

Reviewer #2:

This manuscript describes a method for determining the structures and mechanism of metal

binding oligopeptides by using IM-MS. Some of the results have been published from the same group (e.g., Sesham, R. et al. Eur. J. Mass Spectrom. 2013, 19, 463-473.; Vytla, Y. et al. Anal Chem. 2016, 88, 10925-10932.).

Some specific comments:
-Line 139: What desired pH? "to either pH 3, 4, 5, 6, 7, 8, 9, or 10" has been added
-Line 162-163: The description is confusing and needs rewriting. The sentence has been modified.
-Line 203-204: Check [amb4+3Cu(I)]+, [diamb4+6Cu(I)]2+, [triamb4+9Cu(I)]3+ and [tetraamb4+12Cu(I)]4+ . For example, if [amb4+3Cu(I)] has 3 copper and each copper ion is singly charged, how can the whole complex is singly charged only? Should it be [amb4+3Cu(I)-2H]+? Please supply the mass spectra of these ions as well. They are now shown as "$[amb_4-2H+3Cu(I)]^{+}$, $[diamb_4-4H+6Cu(I)]^{2+}$, $[triamb_4-6H+9Cu(I)]^{3+}$ and $[tetraamb_4-8H+12Cu(I)]^{4+}$." An example of their mass spectra is included as figure 4.
-Figure 4: Please indicate which curve for which ion in a and b. The color indicates which curve is for which ion in a and b.
-Please polish and refine the language. The whole script has been modified and refined.

Reviewer #3:
This manuscript outlines work investigating the use of mass spectrometry to study metal ion binding by peptides. This includes a modified peptide called methanobactin secreted by some methanotrophs to sequester copper, and a set of 'designed alternative metal binding (amb) peptides'. Overall this is not a particularly easy manuscript to follow and the description of the results, and how they address the research aims, need to be much clearer. The connection between the research aims and results has now been included in the new long abstract and in a new section in the introduction. These are re-stated in the experimental, results and discussion sections.

I also have some technical questions about the work. The amb peptides have Cys residues that can readily form disulfide bonds in the presence of oxygen. Were any tests performed to assess the oxidation state of the Cys residues in the apo-peptides and was disulfide bond reduction needed prior to the addition of metal ions? The IM-MS technique includes high resolution TOF m/z analysis and allows the determination of the oxidation state of the Cys. If they are oxidized there is a loss of two hydrogen atoms which is shown in the mass spectrum by a decrease in the mass but not of the charge. At the beginning of each analysis the peptides are run on the IM-MS to make sure the Cys are in their free state and not oxidized. We do not need to use a reduction agent.

Were studies with the peptides in solution performed under anaerobic conditions? We have checked in the past these experiment by running them under anaerobic conditions and get the same results as they are run in aerobic solutions. This work has been published in the past and the reference and statement to this affect given in the text. The addition of Cu(II), and perhaps also other metal ions included in the study, can promote disulfide bond formation and metal reduction. Does this not influence the relevance of the reported studies? Yes, we show that

Cu(II), but not Zn(II), oxidizes the peptide. Our IM-MS analyses can explicitly determine the extent of oxidation and the set of products formed. This is shown in Results section 3. And we have include to new figures (Figs 4 and 5) to show how this is achieved by the IM-MS method.

Work investigating Cu(I) binding is mentioned (section 4.4), but there is no information in the protocol section about how Cu(I), which readily disproportionates in solution unless stabilised, was prepared and handled (only Cu(II) salts are listed in section 1 of the protocol). Cu(I) binding is through the addition of Cu(II) to the peptide. The redox reactions between Cu(II) and the Cys in the peptides form disulfide bonds and Cu(I) binding. This is the interesting result that the IM-MS method can detect. We have include a statement making it clear that the Cu(I)-binding is a result of the addition of Cu(II).

The use of Fe(II) is stated (section 4.4) and reported, but information about the preparation and stabilisation of Fe(II) is also missing (an Fe(III) salt is only listed in section 1 of the protocol). Fe(III) is added to the peptide but Fe(II) binds to the methanobactin. Again we are sure of this because IM-MS measures m/z and can determine the deprotonation state of the mb and the charge state of the metal ion. More details of this have been included in the script.

In many of the results and discussion sections, as well as the abstract, introduction and figures and their legends 'Cu(I/II)' is used. What does this mean (see above)? In other places either Cu(II) or Cu(I) are used. This is really confusing and it is not clear which oxidation state of copper the authors are studying, and I presume it is not possible to distinguish between Cu(II) and Cu(I) binding to a peptide using mass spectrometry? We use Cu(I/II) as a general term because the ambs can bind either Cu(I) or Cu(II) depending on the pH of the solution when Cu(II) is added to them. When we specifically describe a species at a certain pH then we can state whether it is binding Cu(I) or Cu(II). The manuscript has been modified to more clearly show this.

Some specific issues with the results are listed here in the order they appear. In results section 1 the addition of Cu(II) to amb1 is said to give rise to disulfide bond and Cu(I) formation. Why is this pH dependent, and how can the authors be sure about the oxidation state of bound copper? I have modified this section to make this clearer: "Our IM-MS study[20] of amb_1 (**Fig. 1a**) showed that both Cu(I/II) and Zn(II) bound to amb_1 in a pH-dependent manner (**Fig 2**). However, Cu(I/II) and Zn(II) bound to amb_1 through different reaction mechanisms at different coordination sites. For example, adding Cu(II) to amb_1 resulted in oxidation of amb_1 (amb_{1ox}) by disulfide bridge formation and at pH > 6 the $[amb_{1ox}-3H+Cu(II)]^-$ ion (**Fig. 2a**) was formed, indicating the deprotonation of the two imidazoliums, carboxyl group, and two additional sites that were coordinating Cu(II). Molecular modeling of the $[amb_{1ox}-3H+Cu(II)]^-$ ion using B3LYP/LanL2DZ determined the lowest energy complex was Cu(II) coordinated via the imidazole δN of His_1 and the deprotonated nitrogens of the backbone amide groups of Cys_2 and Gly_3. However, below pH 6.0, adding Cu(II) to amb_1 formed a *m/z* isotope pattern that could only be accounted for by Cu(I) binding, forming the $[amb_{1ox}+Cu(I)]^+$ ion (**Fig 2b**), whereas above pH 6 the *m/z* isotope pattern shifted to 1 m/z less accounted by the positive charged $[amb_{1ox}-H+Cu(II)]^+$ ion. Adding Zn(II) did not oxidize amb_1, and Zn(II) binding was observed at

pH > 6.0, primarily forming the $[amb_1{-}3H{+}Zn(II)]^-$ ion (**Fig. 2c**), indicating the deprotonation of the imidazoliums, thiol and carboxyl groups. Molecular modeling of the $[amb_1{-}3H{+}Zn(II)]^-$ ion determined the lowest energy conformers to be either tetrahedral Zn(II) coordination via 2His-2Cys or His-2Cys and the carboxylate of the C-terminus."

In results section 2 the authors say they see 'Cu(I)-binding at low pH transitioned to Cu(II) binding at higher pH' for amb2. Again, how do they know? This is stated in section 1 and the above modification helps explain this. A figure (Figure 4) is now included that shows how we can determine the changing of Cu(I) to Cu(II)-binding as a function of pH.

The situation seems even more complex for amb4 (results section 3). In this section the authors say that 'All these individual species were quantified even though they were chemically similar, which no other analytical technique can achieve in such detail'. Is the complex mixture caused by handling the peptides in air and adding Cu(II) rather than Cu(I)? This section has been modified to address these points and two figures (Figs 4 and 5) have been added to illustrate how Cu(I) is determined from Cu(II) binding and the number of intra- or inter-disulfide bonds are determined.

Copper binding is not mentioned for amb5 as results section 5 (should this be 4?) is all about Zn(II) coordination. Is there a reason for this? The numbering of sections has been corrected. The 2His-2Cys side groups are usually related to Zn(II)-binding so we focused on both Zn and Cu binding for these peptides. In particular we focused on Zn(II) binding for amb_5 and did not consider Cu binding. To keep the article more focused on Cu(I/II)-binding and the comparison between mb-OB3b and the ambs we have removed the amb_5 section on Zn(II)-binding and its figures.

The form of amb7 that best mimics Methylosinus trichosporium OB3b methanobactin (mb-OB3b) will have a single intramolecular disulfide bond. Can this be easily made and metal-ion binding studied? The amb_7 with one disulfide bond is not easily made but we obtained a sample of about 70% purity (based on our high resolution MS analysis) with the other 30% being the amb_7 with no disulfide bonds. Because there is a shift of 2 m/z between the isotope patterns of these two species we can resolve the binding results of the amb_7 (with 1 disulfide bond) from the species with no disulfide bonds using our IM-MS techniques.

Is metal ion binding to amb7 with two intramolecular disulfide bonds relevant (hardly any of the metal ions tested bind to this form of the peptide)? We included $amb_{7\text{-}2}$ to test the how the presence of two free thiols from Cys or no free thiols of Cys affected the binding of these metal ions. However, we have now removed amb7-2 to focus on the species with one disulfide bond.

Cu(I) is mentioned in the results section about mb-OB3b (should this be section 5 and not 6?), but how was Cu(I) binding investigated (I appreciate that Cu(II) is readily reduced to Cu(I) upon addition to mb-OB3b)? Cu(II) was added and we observed Cu(I) binding to the peptide. You can add Cu(I) and you will get the same result as observed for the addition of Cu(II). This has been made clearer in the script.

How is the metal-binding preferences of peptides determined? Is this from the relative occupancy upon the addition of 1 equivalent of the metal ion? In section 4 of the discussion the authors state that mb-OB3b has a preference for Cu(I) over Ag(I). How do they know this from the experiments performed if they added Cu(II)? Yes, this is based on the relative amount of the metal ion added to the relative amount of the metal-bound peptide formed at that titration point. For Cu(II) and Fe(III) all the metal that is bound by the mb-OB3b is either in the oxidation state of Cu(I) or Fe(II).

The first paragraph of the introduction ends with a question about how His and Cys ligands selectively incorporate either Cu(I/II) (again the meaning here is unclear) or Zn(II) to ensure correct function. Can this be addressed in this work given the points raised above, and particularly as many of the Cys residues in the amb peptides readily form disulfide bonds? Yes, as described above we can explicitly determine which oxidation state of Cu and how much is incorporated into the peptide. The IM-MS method can measure the number of disulfide bonds formed during the reactions.

The influence of pH on metal ion binding is discussed in sections 1 and 2. The influence of pH on the Cu(I) affinity of mb-OB3b has been reported (see Inorg. Chem. 2011, 50, 1378-1391) giving a pKa value of 7.0. Is this consistent with the reported studies? The methanobactin peptide has two enethiol oxazolone sites which need to be deprotonated for Cu(I) binding. However, Cu(I) can displace these protons because although there is an order of change in the binding constant from pH 6 to pH 7.5, a binding constant of 10^{19} at pH 6 still ensures quantitative binding of Cu(I). This is consistent with our binding studies conduct at pH 6 which shows quantitative binding of Cu(I). However, the ambs with His and Cys show far greater pH dependence, where quantitative binding is only observed at pH> 9. This has been added in the discussion.

Sections 2 and 4 of the discussion are particularly unclear about the oxidation state of copper. In section 4 there is mention of the high reduction potential of Cu-mb-OB3b and the authors say this 'supports a wide range of adventitious reducing partners to form mb-OB3b+Cu(I)'. What does this mean? This sentence has been modified. The reduction potential of Cu-mb-OB3b was published in 2011 (Inorg. Chem. 2011, 50, 1378-1391) and no other group has reported the reduction potential of any mb (other mbs have had their reduction potentials measured - see PNAS 2012, 109, 8400-8404 and Chem. Eur. J. 2018, 24, 4515-4518, and there are quite large variations). The two papers cited in the manuscript about the reduction potential of Cu-mb-OB3b (references 23 and 48) are from 2008 and 2010, before the value had been reported in the literature. Reference 47 seems to have nothing to do with Cu(I) coordination in mb-OB3b (section 4 of the discussion). The references have been removed and the sentence changed to "Cu(II) reduction was also mediated by thiol oxidation and disulfide bridge formation in contrast to the existing disulfide bridge in apo-mb-OB3b and the high reduction potential for copper-loaded mb-OB3b, which supports the strong preference for Cu(I).[47] " which now references Inorg. Chem. 2011, 50, 1378-1391. A key review about methanobactins (Microbiol. Mol. Biol. Rev. 2016, 80, 387-409) could be cited. In summary, use of the correct literature needs to be thoroughly checked throughout this manuscript by the authors. The review article has been

included in the introduction describing mb and the other references have been checked and corrected.

Some other more minor points in the order they appear
The second sentence of the introduction is long and it is not clear what 'them' is referring to in 'selectively incorporate them'. In the same sentence enzymes, or maybe metalloenzymes, could be used in place of 'enzymatic proteins'. The sentence has been shortened and modified: " To enable these functions, groups such as the thiolate of Cys, imidazole of His[2,3] or, more rarely, thioether of methionine, and carboxylate of Glu and Asp selectively incorporate metals as cofactors into the active sites of metalloenzymes."

The relevance of this work to copper-related diseases is not clear. mb-OB3b has potential to treat patients with Wilson's disease, but will the studies described in this manuscript, and particularly those about the amb peptides, make a contribution in this area? Therefore, does the introduction need to include so much information on this topic and is the closing sentence of the abstract appropriate? The abstract has been changed to describe the instrumental and theoretical methods and the introduction modified to include more about the instrumental method while keeping the relevance to chelating peptides as potential therapeutics.

Does the binding of metals other than copper to mb-OB3b have any physiological relevance? If not, this point should be made. The following has been added to the introduction "Competition for the physiological Cu(I) binding site was mainly from Ag(I) because it was able to displace Cu(I) from the complex, and both Ag(I) and Ni(II) could irreversibly bind to Mb and not be effectively displaced by Cu(I).[19]"

M. trichosporium OB3b was cultured not mb-OB3b (protocol). Thank you, this has been corrected.

Nickle(II) chloride should be changed to nickel(II) chloride (protocol). changed.

The mb-OB3b used in this work is quoted as being only 35.0% pure. How was this value determined using a titration with Cu(II), and what does it actually mean? How precise is this value as a 'purity correction' has been applied in all of the mb-OB3b experiments? Why is the mb-OB3b sample so impure? Mb-OB3b quantitatively binds Cu(II) as Cu(I) at pH 6.5. Therefore, the IM-MS titration with known moles of Cu(II) was added to known amount of mb-OB3b and the end-point, where all mb-OB3b was converted to the Cu(I)-bound mb-OB3b, was used to determine its purity at 35.0%. This purity correction was precise to 3 sig. figs, and all the titrations of the mb-OB3b sample were corrected by it. The protocol has been edited to include some more detail. Dr. Choi who is an expert of mb-OB3b purification techniques, purified the mb-OB3b from the *M. trichosporium* media and was not surprised by the purity we determined.

The numbering of the results, after section 3, needs correcting. Yes, this has been done.

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