Mass Spectrometry and Metallomics: a General Protocol to Assess Stability of Metallodrug-Protein Adducts in Bottom-Up MS Experiments

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ABSTRACT: The bottom-up mass spectrometry approach is today one of the best tools of Metallomics to characterize the binding of metal-based drugs to proteins. Yet, the stability of metal-protein coordination bonds along the whole process may be a critical issue. This led us to build up a general protocol to test metallodrug-protein adduct stability under the typical conditions of the filter-aided sample preparation (FASP)/bottom-up procedure, ranging from the analysis of solutions containing metal-protein adducts to tandem mass spectrometry experiments. More in detail, we identified nine critical situations, either during the sample manipulations or instrumental, as a potential source of metal-protein bond impairment when using FASP operative conditions and a nano high performance liquid chromatography-nanoelectrospray ionization-LTQ-Orbitrap (nanoLC-nanoESI-LTQ-Orbitrap) mass spectrometer system, equipped with a preconcentration/purification device. These are: 1) sample permanence in the ammonium bicarbonate buffer; 2) denaturation with urea; 3) reduction with dithiothreitol; 4) alkylation with iodoacetamide; 5) sample permanence in the loading mobile phase; 6) sample permanence in the elution mobile phase; 7) the nanoESI process; 8) the transfer of the adduct through the ion transfer tube and tube lens; 9) collision induced dissociation in the ion trap. Accordingly, an *ad hoc* experimental protocol was developed and applied to the adducts formed between cytochrome *c* (Cyt *c*) and two different metallodrugs, *i.e.* cisplatin (*cis*-diamminedichloridoplatinum(II), CDDP) and RAPTA-C, a well-known ruthenium(II)-arene compound [Ru(η^6-*p*-cymene)Cl_2(pta)] (pta = 1,3,5-triaza-7-phosphaadamantane), used here as models. Notably, Cyt *c*-CDDP adducts were stable through all the above conditions while Cyt *c*-RAPTA-C adducts turned out unstable in the ammonium bicarbonate buffer. This latter finding supports the need to perform a test-protocol of this kind when starting any extensive bottom-up MS investigation of protein-metallodrug systems.

1. Introduction

Since the discovery of the antitumor properties of cisplatin (Fig. 1) by Rosenberg and co-workers [1] during the sixties, metal based drugs have been playing a major role in anticancer chemotherapeutic strategies. There is today a general consensus on the necessity to elucidate the mechanism of action of metal based drugs at the molecular level in such a way to rationally design novel and better anticancer metallodrugs through the so called "mechanism oriented" approach. In general, DNA is considered as the primary target for CDDP and its close analogues [2,3] while proteins appear to play crucial roles in the transport, uptake, excretion, biodistribution, toxicity profile and resistance phenomena related to Pt drugs themselves. Even more interesting, proteins are involved in crucial aspects of the mode of action of various non-platinum anticancer agents, like ruthenium complexes [4].

Metallomics is mainly concerned with the identification and characterization of all chemical species, present in a certain biological sample (a cell, a tissue or an organism) that contain the metal of interest, e.g. Pt or Ru. To study metallodrug-protein interactions in more depth, researchers can take advantage of

the availability of very sophisticated and advanced analytical tools, the main being X-ray diffraction, nuclear magnetic resonance (NMR) and mass spectrometry (MS).

MS represents today a fast, sensitive, specific and high-throughput tool for the analysis of biomolecules; in particular, electrospray ionization mass spectrometry (ESI-MS) potentially provides a wealth of structural and functional information mainly due to its non-destructive nature that even preserves non-covalent interactions [5]. A series of pioneering studies carried out by Dan Gibson and coworkers [6,7] during the 1990s and the early 2000s highlighted the advantages of this method and defined the best experimental conditions for its application to metallodrug-protein systems; extensive information on the chemical nature of the adducts (metallo fragment-protein, being the metallo-fragment the part of the metallodrug that remains attached to protein), on binding stoichiometry and binding site location can thus be derived making MS one of the most effective tools of modern metallomics.

In general, for the characterization of metallodrug-protein binding sites using MS techniques, two possible strategies can be exploited: the so-called “top-down” and “bottom-up” approaches.

In the top-down approach the whole metallodrug-protein adduct is directly fragmented, avoiding further treatments and so reducing the possibility to lose the metal fragments attached to the protein during those processes and manipulations. However, its application generally requires the use of high resolution instruments and is restricted to relatively small proteins, mainly because of the highly complex mass spectra that are generated.

On the other hand, the classical bottom-up approach is better used for high molecular weight proteins because it involves sample reduction, alkylation and enzymatic digestion prior to LC-ESI-tandem mass (MS/MS) analysis. However, each of these preparation/analytical steps might compromise the stability of the metallodrug-protein adduct. Therefore, although this kind of approach has already been applied in many studies, the preparation/analytical conditions must be carefully settled in order to ascertain their suitability to preserve metal-protein binding along the whole process. For example, Moreno-Gordaliza et al. [8] studied in depth the effect of denaturing (urea), reducing (dithiothreitol, DTT) and alkylating (iodoacetamide, IAA) reagents in the presence of Tris buffer during routine procedure for in solution tryptic digestion of CDDP-insulin adducts. The same authors [9] tested the stability of the adducts formed between CDDP and five model proteins along the whole in-gel digestion protocol. In turn, Moraleja et al. [10] focused their investigations on the comparison of two reducing agents, DTT and tributylphosphine (TBP), used in the FASP-digestion procedure for CDDP-protein adducts.

In any case, the reagents typically used in the enzymatic digestion are not the only triggers of metal-protein coordination bond impairment. Karas et al. [11] reported the sensitivity of the coordination bond between Fe-heme and histidine in myoglobin (Mb) to some mass spectrometer instrumental parameters (capillary temperature, capillary/skimmer voltage) while Li et al. [12] and Loo [13] described its sensitivity to pH, to the presence of organic co-solvents and to induced collision dissociation (CID). Also, Will et al. [14] listed the requirements that must be fulfilled by CDDP-protein adducts for a subsequent multidimensional protein identification technology (MudPIT)/MS/MS analysis (kinetic stability over the range $2.3 < pH < 8.5$, metallo fragment persistence on MS/MS fragments).

These arguments convinced us to design a general protocol to test, a priori and systematically, metallodrug-protein adduct stability under the conditions of the FASP/bottom-up mass spectrometry approach, starting from the digestion process, passing through the nanoLC step and ending with nanoESI-MS/MS analysis in a LTQ-Orbitrap mass spectrometer. In the present article, we describe details of this protocol and apply it to two model systems, both containing Cyt *c*, an important protein crucially involved in apoptotic pathways [15], commercially available and easily characterisable by ESI-MS analysis. For the first system, the well-known CDDP antitumor drug was chosen while, for the second one, the less investigated RAPTA-C (Fig. 1), a Ru(II) complex mainly characterized by limited direct cytotoxic effects on cancer cell in vitro and an anti-metastatic behavior in vivo [16] was selected.

2. Experimental

2.1 Solvents, reagents and materials

Water (412091) and acetonitrile (412042), both UHPLC-MS grade, were purchased from Carlo Erba (BP 616, F-27106, Val de Reuil Cedex, France). DMSO (8.02912.1000) was purchased from Merck Schuchardt OHG (Hohenbrunn, 85662, Germany). Ammonium acetate (AA, A1542), ammonium bicarbonate (Ambic, 09830), DTT (43815), IAA (57670), HCOOH (56302) and TFA (40967) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and urea from Serva (Heidelberg, Germany). Microcon centrifugal filter devices with nominal cutoff of 3 kDa (42404) were purchased from Millipore (Bedford, MA 01730, USA).

2.2 Metal complexes, peptides and proteins

CDDP (P-4394), MRFA tetrapeptide (M1170), equine skeletal muscle Mb (M0630) and horse heart Cyt *c* (C7752) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RAPTA-C was prepared according to literature [17,18]. The EHSG tetrapeptide was synthetized and purified following the experimental protocol previously described [19].

2.3 Mass spectrometric analyses and CID experiments

ESI-MS spectra were recorded by direct introduction of sample solutions at 5 µl/min flow rate into a LTQ-Orbitrap high resolution spectrometer (Thermo, San Jose, CA, USA or Thermo Fisher, Bremen, Germany), equipped with a conventional ESI source. The working conditions, if not differently specified in "Results and discussion" or "Supplementary Information" paragraphs, were the following: positive polarity, sheath and auxiliary gases, respectively, 17 and 1 a.u. (arbitrary units), spray voltage 3.1 kV, capillary voltage 45 V, capillary temperature 220 °C, tube lens voltage 230 V, nominal resolution (at m/z 400) of 100,000. CID experiments were performed in ion trap (IT) analyser (30% relative collision energy) and the related spectra were acquired in FT analyser (nominal resolution 100,000 at m/z 400). Isolation widths used are specified in "Results and discussion" paragraph. Xcalibur 2.0 software (Thermo) was used for acquisition and deconvoluted spectra were obtained by using the integrated Xtract tool.

2.4 Holo-Mb: solution preparation and study of protein stability to denaturing conditions and instrumental parameter values

Holo-Mb solutions were prepared at 5 µM concentration in H_2O, H_2O/CH_3CN 1/1 or H_2O/CH_3CN 1/1 with 0.1% HCOOH (CH_3CN and HCOOH just added before ESI-MS analyses). Only the aqueous solution was analysed at different values of capillary temperature, capillary voltage and tube lens voltage (specified in "Result and discussion" or "Supplementary Information" paragraphs).

2.5 Cyt c-CDDP and Cyt c-RAPTA-C adduct formation (mother solutions)

Cyt *c*-CDDP adducts were prepared by mixing 500 µl of 100 µM Cyt *c* solution in 20 mM AA buffer at pH 6.8 with 5 µl of $3 \cdot 10^4$ µM CDDP solution in DMSO (1:3 protein-to-metal ratio) and incubating for 114 h at 37 °C (mother solution). After this time, two aliquots, of 25 µl each, were withdrawn from the mother solution and diluted with 475 µl of H_2O to a 5 µM Cyt *c* final concentration. Only one, of these two diluted aliquots, was purified on centrifugal filter device: 500 µl of the diluted solution were spinned for 1 hour at 13000 rpm, washed with 250 µl of H_2O, spinned again for 30 min at 13000 rpm

and the volume recovered, by spinning the filter upside down, was diluted at 500 µl with H_2O. The purified and the unpurified diluted aliquots were analysed by ESI-MS. The remaining mother solution was freezed at -20 °C and subsequently used. For the preparation of Cyt *c*-RAPTA-C adduct mother solution an analogue procedure was followed with the only differences that Cyt *c* and RAPTA-C were solubilized in H_2O and incubated for 72 h.

2.6 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability at denaturation, reduction, alkylation steps in Ambic aqueous solution

An aliquot of 25 µl of mother solution was purified from the metal complex excess on centrifugal filter device (25 µl of mother solution were placed on the centrifugal filter device together with 200 µl of Ambic 50 mM, they were spinned for 45 min at 13000 rpm, washed again with 200 µl of Ambic 50 mM and spinned again for 45 min at 13000 rpm). The concentrate was then denatured on centrifugal filter device (200 µl of urea 8 M in Ambic 50 mM were added and the filter was spinned for 45 min at 13000 rpm; this procedure was repeated once and then 200 more µl of urea 8 M in Ambic 50 mM were added). For the reduction step 2 µl of a freshly prepared 0.5 µg/µl DTT aqueous solution (15:0.5 protein-to-DTT ratio, w/w) were added directly on the centrifugal filter device and the sample was incubated for 30 min at room temperature (rt). For the alkylation step 2 µl of a freshly prepared 2.5 µg/µl IAA aqueous solution (15:2.5 protein-to-IAA ratio, w/w) were added directly on the centrifugal filter device and the sample was incubated for 20 min at rt in the dark. The sample was then purified from urea, DTT and IAA (the sample was spinned for 45 min at 13000 rpm and twice washed with 200 µl of Ambic 50 mM and spinned for 45 min at 13000 rpm). The concentrate was then diluted with 200 µl of Ambic 50 mM and finally incubated overnight on filter at 37 °C. The morning after Ambic was removed (the sample was spinned for 45 min at 13000 rpm on centrifugal filter device, washed with 150 µl of H_2O and spinned again for 30 min at 13000 rpm) and the concentrate recovered by spinning the filter upside down at 3500 rpm for 3 min was diluted at 500 µl (approximately 5 µM Cyt *c* final concentration) with H_2O just before ESI-MS analysis.

2.7 Cyt c-RAPTA-C adduct stability in Ambic aqueous solution

An aliquot of 25 µl of mother solution was purified from the metal complex excess on centrifugal filter device (25 µl of mother solution were placed on the centrifugal filter device together with 200 µl of Ambic 50 mM, they were spinned for 45 min at 13000 rpm, washed again with 200 µl of Ambic 50 mM and spinned again for 45 min at 13000 rpm). The concentrate was then diluted on filter with 200 µl of Ambic 50 mM and finally incubated overnight at 37 °C. The morning after, Ambic was removed (the sample was spinned for 45 min at 13000 rpm on centrifugal filter device, washed with 150 µl of H_2O and spinned again for 30 min at 13000 rpm) and the concentrate recovered by spinning the filter upside down at 3500 rpm for 3 min was diluted at 500 µl (approximately 5 µM Cyt *c* final concentration) with H_2O just before ESI-MS analysis.

2.8 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability in loading mobile phase

An aliquot of 25 µl of mother solution was diluted with 475 µl of CH_3CN/H_2O 1/1 with 0.1% TFA to a 5 µM Cyt *c* final concentration and the resulting solution was analyzed as such in ESI-MS after 5 and 20 min of incubation at rt.

2.9 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability in nano mobile phase

An aliquot of 25 µl of mother solution was diluted with 475 µl of CH_3CN/H_2O 1/1 with 0.1% HCOOH to a 5 µM Cyt *c* final concentration and the resulting solution was analyzed as such in ESI-MS after 5, 20 and 45 min of incubation at rt.

2.10 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability in ESI source

Aliquots of 12.5 µl were withdrawn from Cyt *c*-CDDP solution at different incubation times at 37 °C (0 h, 24 h, 48 h, 72 h, 144 h) and diluted with 237.5 µl of CH_3CN/H_2O 1/1 with 0.1% HCOOH to a 5 µM Cyt *c* final concentration. The resulting solutions were analysed as such in ESI-MS. For Cyt *c*-RAPTA-C solution an analogue procedure was followed at the incubation times 0 h, 24 h, 48 h, 72h.

2.11 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability to instrumental parameters

An aliquot of 25 µl of mother solution was diluted with 475 µl of CH_3CN/H_2O 1/1 with 0.1% HCOOH to a 5 µM Cyt *c* final concentration and the resulting solution was analysed as such in ESI-MS at various values of capillary temperature, capillary voltage and tube lens voltage (specified in "Results and discussion" or "Supplementary Information" paragraphs).

2.12 Peptide, peptide-CDDP and peptide-RAPTA-C solution preparation

Aqueous solutions (100 µM) were prepared for the two peptides EHSG and MRFA, aliquots were diluted with CH_3CN/H_2O 1/1 with 0.1% HCOOH to a peptide final concentration, respectively, of 50 µM and 2.5 µM and the resulting diluted solutions were immediately analysed in ESI-MS with full scan and CID experiments.

MRFA-CDDP and EHSG-CDDP adducts were prepared by mixing 500 µl of 100 µM peptide aqueous solution with 5 µl of $3 \cdot 10^4$ µM CDDP solution in DMSO (1:3 peptide-to-metal ratio) and incubating at 37 °C for 72 h or 24 h, respectively, for MRFA or EHSG.

MRFA-RAPTA-C and EHSG-RAPTA-C adducts were prepared with analogue procedure: 5 µl of $3 \cdot 10^4$ µM RAPTA-C aqueous solution were added to 500 µl of 100 µM peptide aqueous solution (1:3 peptide-to-metal ratio) and the resulting mixtures were incubated at 37 °C for 24 h.

Aliquots of these peptide-CDDP or peptide-RAPTA-C adduct solutions were diluted with CH_3CN/H_2O 1/1 with 0.1% HCOOH to a 50 µM peptide final concentration and immediately analysed as such in ESI-MS with full scan and CID experiments.

3. Results and discussion

3.1 Holo-Mb: sensitivity of the coordinate bond between Fe-heme and histidine to organic cosolvents and pH, capillary temperature, capillary voltage and tube lens voltage values

At first, for comparison purposes, we carried out some experiments on the previously described holo-Mb system. For holo-Mb, as already reported in the literature [11,12,13], the stability of the coordinative bond between Fe-heme and histidine may be heavily affected by many solution (presence of an organic cosolvent, pH values) and instrumental (capillary temperature, capillary/skimmer voltage, CID) conditions.

We decided to test again some of these conditions to better define the optimal instrumental setting to be applied to the analysis of metal-protein adducts. Accordingly, holo-Mb solutions were infused in an LTQ-Orbitrap mass spectrometer by using different values for the instrumental parameters (see Fig. 2 and Fig. S1 in the ESI). Results confirmed that the addition of an organic cosolvent (50% CH_3CN, Fig. 2b) to the aqueous solution (Fig. 2a), and the further addition of 0.1% HCOOH (Fig. 2c), destroy the coordination bond between Fe-heme and histidine: indeed, the peak assigned to holo-Mb, at 17567 Da, disappeared almost completely or completely (see Fig. 2b and Fig. 2c) being replaced by the peak of apo-Mb at 16950 Da. Among the several instrumental parameters, we decided to test those considered

most critical in tuning optimization of an LTQ-Orbitrap instrument, i.e. capillary temperature, capillary voltage and tube lens voltage. By decreasing both capillary temperature and tube lens voltage, from 220 °C and 230 V, respectively, to 175 °C and 140 V (compare Fig. 2a and Fig. 2d) the peak of apo-Mb was no more detected; in contrast upon increasing them to 275 °C and 250 V (compare Fig. 2a and Fig. 2e) the apo-Mb peak increased its relative intensity. Instead, changes imposed to capillary voltage turned out not to be critical (see Fig. S1 in ESI) for apo-Mb formation.

From the previous results the instrumental conditions reported in Fig. 2d (capillary temperature 175 °C, tube lens voltage 140 V, capillary voltage 45 V) turned out to be optimal to maximize holo-Mb and thus, in principle, other metallo-protein adducts. On the other side, we believe that conditions of Fig. 2a (capillary temperature 220 °C, tube lens voltage 230 V, capillary voltage 45 V) are the best compromise between metal-protein adduct preservation and signal intensity during infusions in our LTQ-Orbitrap instrument.

3.2 Critical issues for metallo drug-protein adduct stability in the FASP/bottom-up approach using nanoLC-nanoESI-LTQ-Orbitrap-MS/MS analysis: the "nine-point testing protocol"

The literature results and the findings reported in the previous paragraph, as well as the already mentioned problems (see Introduction) related to denaturation, reduction, alkylation steps, prompted us to draw up a general and systematic protocol to test, a priori, metallo fragment-protein adduct stability during the whole bottom-up mass spectrometry approach. In fact, the nature of the metal, of its ligands and the protein micro-environment are variables that can affect deeply and unpredictably the resistance of the metal-protein coordination bond during the bottom-up approach: nothing can be said a priori about this.

The method we intend to use for future studies of binding site location will take into consideration the denaturation, reduction, alkylation, digestion procedure and nanoLC-nanoESI-MS/MS analysis that we usually apply in our laboratories for shotgun proteomic experiments [20], the only difference being the presence of an on-line purification/concentration cartridge to avoid sample direct injection on column. We also intend to combine this procedure with the FASP [21] one since the latter limits the contact time between denaturing/reducing/alkylating agents and the metallo fragment-protein adduct, thus avoiding possible side reactions causing metal loss.

In Scheme 1 we list, step by step, the bottom-up procedure we intend to follow (blue rectangles) and, in parallel, we highlight the nine critical conditions we identified as potential sources of metal-protein coordination bond impairment (red rectangles) and the experimental conditions we tested in this article (violet rectangles). In our opinion, these nine points, -the "nine-point testing protocol"-, constitute a useful and almost complete track (though this protocol is obviously open to additions and changes, according to the chosen instrument and the applied sample preparation procedures) to be followed whenever one intends to study the stability of metallo fragment-protein adducts throughout the whole bottom-up process. The protocol reveals its utility particularly in the case of less studied, or completely new, metal complexes; pairwise, we suggest to use it to test the same metal complex incubated with different proteins.

3.3 Metallodrug solution preparation

CDDP and RAPTA-C solutions were both prepared at the concentration of $3 \cdot 10^4$ μM. RAPTA-C was easily solubilized in water while, to reach that concentration, 9 mg of CDDP were dissolved in 1 ml of DMSO (according to Merck Index, CDDP solubility in H_2O is only 2.53 mg/ml at 25 °C). The formation of Cyt *c*-CDDP and peptide-CDDP adducts containing DMSO as ligand is, in this case, predictable. This kind of adducts have no clinical/biological relevance but, since the aim of this paper is to propose a methodologic approach to test adduct stability in general and to highlight their potential

different behavior during bottom-up experiments, we decided to consider them appropriate for our purposes.

3.4 Cyt c-CDDPt and Cyt c-RAPTA-C adduct stability at denaturation, reduction, alkylation steps in Ambic aqueous solution (points 1-4)

Cyt *c*-CDDP and Cyt *c*-RAPTA-C solutions were subjected to purification (centrifugations and washings with Ambic 50 mM) on centrifugal filter device to remove the excess of unreacted metal complex before adding urea, DTT and IAA. This step avoids undesired side reactions between the free metal complex and these three reagents. The solutions were subsequently denatured, reduced, alkylated directly on filter and then purified from urea, DTT, IAA by centrifugations and washings with Ambic 50 mM. Finally, the solutions were incubated overnight on filter at 37 °C to simulate tryptic digestion. The day after Ambic was washed away with H_2O and the samples, diluted to the desired concentration with H_2O, were analyzed by ESI-MS.

Cyt *c*-CDDP adducts were stable (see Fig. 3) toward Ambic, urea, DTT and IAA in the times and quantities specified in the first violet rectangle of Scheme 1, exactly the same conditions that will be used in the bottom-up approach (first red rectangle): indeed, no appreciable changes are highlighted in the relative intensities, respectively, of the peak of unreacted Cyt *c* (12358 Da) and its mono-, bis- and tris-adducts.

Instead, Cyt *c*-RAPTA-C adducts turned out to be unstable in the same conditions (see Fig. 4a-b): indeed, after treatment with Ambic, urea, DTT and IAA the mono- and bis-adducts Cyt c + $[Ru(\eta^6\text{-}p\text{-cymene})]^{2+}$ at 12591 Da, Cyt *c* + $[Ru(\eta^6\text{-}p\text{-cymene})(pta)]^{2+}$ at 12748 Da and Cyt *c* + $[Ru(\eta^6\text{-}p\text{-cymene})(pta)]^{2+}$ + $[Ru(\eta^6\text{-}p\text{-cymene})]^{2+}$ at 12982 Da almost or completely disappeared while Cyt *c* at 12358 Da became the most intense peak in the spectrum.

A deeper investigation was carried out on Cyt *c*-RAPTA-C adduct stability in Ambic using the same procedure described above but leaving out the denaturation, reduction and alkylation steps. Interestingly the results showed adducts disappearance in Ambic 50 mM (Fig. 3c), thus indicating that the use of this latter buffer poses a stability issue. Gibson et al. already reported on the sensitivity of ubiquitin-CDDP adducts to Ambic [6] and our outcomes confirmed that an accurate evaluation of this crucial parameter must be always taken into account, especially in light of the different results obtained for Cyt *c*-CDDP and Cyt *c*-RAPTA-C: indeed, the same protein, incubated with different metal complexes, gives rise to adducts of different stability in Ambic 50 mM.

In order to explain such different behavior we believe appropriate to discuss, first of all, the CDDP and RAPTA-C binding site location on Cyt *c*.

According to HSAB (hard and soft acids and bases) theory, the S-donors present in the amino acid side chains of proteins would be the preferential binding sites for Pt^{2+} complexes but, in the literature, many examples are reported which seem to contradict this expectation. In fact, there is a close correlation between the anchoring position and the incubation conditions used. The case of the CDDP/human transferrin (hTrfe) system is significant in that sense: if the incubation is conducted in a 1:1 ratio at 37 °C for 24 h, Met256 and Met499 are identified as binding sites in NMR experiments [22], while T457 is identified in ESI-MS/MS experiments if using a 10:1 CDDP/hTrfe ratio, 20 °C and 30 min of reaction [23, 24]. This suggests that residues that contain O-donors could offer kinetically preferred binding sites for CDDP in hTrfe, but Met residues might gain in importance after prolonged incubation. Regarding the specific model system used in this article we found a similar situation. Met65 is generally accepted as the primary CDDP binding site on Cyt *c*, as shown by Zhao et al. with ESI-MS experiments [25] and by Ferraro et al. in X-ray diffraction experiments [26]. However, according to the results coming from liquid chromatography coupled with LTQ-MS [9] and Fourier transform ion cyclotron resonance mass spectrometry [27], other S-, O- and N-donors, besides Met65, resulted as

possible binding sites (respectively Met80, Glu61, Glu62, Thr63 in the first case and Met80, His18, His 33 in the second case).

Similar observations can be made for Ru compounds. A statistical analysis, carried out on crystallographic structures of Ru complexes directly bound to protein residues, reveals that Ru ions mainly bind His side chains on both NE2 and ND1 [28]. However, in the same review, other residues involved in the Ru compound-protein recognition are reported: Glu, Asp, Cys, Lys, Asn, Gln, Arg. The strong preference of Ru(II) complexes for the N-donor His has already been reported also for the specific system RAPTA-C/Cyt *c* [29].

In the light of the above discussion it seems reasonable to establish that Met65 and His33 are the main targets of, respectively, CDDP and RAPTA-C on Cyt *c*. The different behavior of these two putative adducts in Ambic 50 mM is not easy to explain and we believe that this issue needs to be studied in depth with further experiments.

Finally, we have to underline that Cyt *c*-RAPTA-C adduct instability in Ambic does not rule out further instability to urea, DTT and IAA reagents but, at this level, we decided to investigate no longer on this issue. In particular, for DTT, it has been already highlighted [9] how the disappearance of metal-protein adducts is probably related to the initial amount of the latter in the original sample, to their binding strength and their location in the protein. Moreover, it should be noted that Cyt *c* would not need reduction-alkylation since its unique two cysteines are covalently involved in heme *c* binding. Nevertheless, in this article, we decided to perform these two steps in order to show the use of the "nine-point testing protocol" in its entirety.

3.5 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability in loading and nano mobile phases (points 5-6)

The stability of Cyt *c*-CDDP and Cyt *c*-RAPTA-C adducts versus organic cosolvent and acidity present in the loading and nano mobile phases was studied. Liquid chromatography loading step on an on-line preconcentration/purification cartridge involves sample permanence in H_2O/CH_3CN 98/2 with 0.1% TFA for 3 min (Scheme 1, second red rectangle). Instead, sample elution from nano column involves the use of different H_2O/CH_3CN ratios, ranging from 4% to 65% of CH_3CN, with 0.1% HCOOH for 35 min (Scheme 1, third red rectangle). To evaluate Cyt *c*-CDDP and Cyt *c*-RAPTA-C resistance to these conditions we diluted mother solutions (see Experimental section) in CH_3CN/H_2O 1/1 with 0.1% TFA or CH_3CN/H_2O 1/1 with 0.1% HCOOH and analyzed the resulting samples as such by ESI-MS after different incubation times at rt (Scheme 1, second and third violet rectangles). At this level the excess of metal compound was not removed from solutions before ESI-MS analyses: previously performed experiments (data not shown) demonstrated the possibility to skip this purification step without the risk of incurring in the formation of aspecific adducts. This purification step was also omitted during point 7 and 8.

The spectra reported in Fig. 5 clearly show the stability of Cyt *c*-RAPTA-C adducts in CH_3CN/H_2O 1/1 with 0.1% HCOOH at the tested times: the relative ratios between unreacted Cyt *c* and its mono- and bis-Ru adducts remains costant in the range time 0-45 min. Almost analogous results were obtained for the same adducts in CH_3CN/H_2O 1/1 with 0.1% TFA (Fig. S2 in the ESI) and for Cyt *c*-CDDP adducts in CH_3CN/H_2O 1/1 with 0.1% HCOOH or 0.1% TFA (see, respectively, Fig. S3 and S4 in the ESI). It is important to notice that the conditions experimentally applied to test stability at loading mobile phase are harsher than those actually used in the bottom-up procedure (compare the second red and violet rectangles in Scheme 1). Instead, the experimental conditions applied to test adduct stability in the nano mobile phase are characterized by longer incubation times and slightly lower CH_3CN percentage than those reached during bottom-up analysis (compare the third red and violet rectangles in Scheme 1).

Actually, it would be more appropriate to apply the stability tests described in this section to metallated peptides instead of protein adducts since, during the bottom-up approach, digested metallated

peptides come into contact with mobile phases. However, it would be rather complicated and expensive, respectively, to foresee and synthesize all these peptides and therefore, in our opinion, this stability test performed on denatured protein adducts turns out to be a good compromise.

Finally, it is interesting to notice the different behavior between Mb, on one side, and Cyt *c*-CDDP and Cyt *c*-RAPTA-C adducts, on the other, with respect to the presence of an organic cosolvent and acidity (compare Fig. 2a-c with Fig. 5, S2, S3, S4): the coordination bond between Fe-heme and histidine in Mb showed its sensitivity to CH_3CN and low pH values while Pt and Ru adducts on Cyt *c* demonstrated a high resistance to those conditions. These findings again confirm how the nature of the metal as well as the strength and the position of the binding can affect adduct stability.

3.6 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability in nanoESI source (point 7)

During the ESI process, metal-protein adduct dissociation may occur: the mere transfer of the adduct from the solution to the gas phase, as well as the presence of an inert gas (nitrogen), can induce the detachment of the metal centre [30]. In order to verify the stability of Cyt *c*-CDDP and Cyt *c*-RAPTA-C adducts during ESI process (absence of false negatives), aliquots were withdrawn from the two solutions at different incubation times, were diluted/denatured in CH_3CN/H_2O 1/1 with 0.1% HCOOH and then analyzed as such by ESI-MS. The non-destructive nature of conventional ESI source was deduced by following the adduct formation kinetic (see Fig. 6 and Fig. S5 in the ESI): the intensity of peak adducts grows congruently with time and, mutually, unreacted Cyt *c* peak intensity decreases.

By the same experimental evidences, we also deduced the absence of aspecific bond formation (false positives) between Cyt *c* and CDDP (or RAPTA-C) in conventional ESI source.

A fortiori the conservativeness of the nanoESI source, that is used in bottom-up approach (see the fourth red rectangle in Scheme 1), will be ensured: nanospray is believed to be more gentle than conventional electrospray since lower voltage are applied and there is no presence of nitrogen (therefore there are fewer activating collisions in the nanospray source [31]).

As already reported in the previous paragraph, it would be more appropriate to apply this stability test to metallated peptides instead of protein adducts but, for the same reasons explained above, its application to denatured protein adducts results to be a good compromise.

3.7 Cyt c-CDDP and Cyt c-RAPTA-C adduct stability to instrumental parameters (point 8)

Cyt *c*-CDDP and Cyt *c*-RAPTA-C adduct stability to capillary temperature, capillary voltage and tube lens voltage variations was studied by diluting the mother solutions (see Experimental section) in CH_3CN/H_2O 1/1 with 0.1% HCOOH and analysing the resulting solution by ESI-MS. At variance with Mb (see Fig. 2a, 2d, 2e), Cyt c-CDDP and Cyt c-RAPTA-C adducts showed their complete stability to the variations imposed to capillary temperature and tube lens voltage (see Fig. 7 and S6 in ESI) and, similarly to Mb, they proved their stability to capillary voltage variations (compare Fig. S1 with Fig. S7 and S8).

For the same reasons already explained in the previous two paragraphs, also these stability tests were performed on denatured protein adducts instead of metallated peptides.

3.7 Metallo fragment-pepdide adduct stability at CID (point 9)

Experiments were carried out to evaluate the stability at CID of metallo fragment-peptide adducts generated during the enzymatic digestion of metallo fragment-protein adducts. This test aim to investigate the resistance to CID not only of Pt-peptide (or Ru-peptide) bond but also of Pt-ligand (or Ru-ligand) one. In fact, the energy provided during MS/MS experiments for peptide chain sequencing could generate, in addition, breakage of metal-peptide and metal-ligand bonds. In particular, the study of this last type of fragmentation provides important information to be used in the design of targeted

MS/MS experiments [32] and in the subsequent reworking of bottom-up results through search engines [33], like Sequest and Mascot, or by manual interpretation [21].

From an experimental point of view the above-mentioned stabilities were evaluated by using a simplified system consisting of CDDP, or RAPTA-C, incubated with model peptides. The choice of the model peptides was done by taking into account the already discussed (see paragraph 3.4) high affinity of Pt(II) and Ru(II) complexes for S- and N-donors contained in amino acid side chains. In this article, in order to explain how to deal with point 9) of the testing protocol, we selected the model tetrapeptides MRFA, containing Met, and EHSG, containing His, leaving out, in the first instance, other possible S-, N- and O-donors. Obviously, for a general applicability of such test, the nature of the model peptides will have to be modulated, from time to time, depending on the specific nature of the metal complex under examination.

In each of the four incubation mixtures (CDDP-MRFA, CDDP-EHSG, RAPTA-C-MRFA, RAPTA-C-EHSG) a specific Pt-peptide, or Ru-peptide, adduct ion was selected for CID experiments (see Fig. S9 and S10 in ESI). Fig. 8 and Fig. 9 show the CID spectra of the four selected peptides, namely $[MRFA+Pt^{2+}-H^+]^+$ at 717.22 m/z, $[EHSG+Pt(NH_3)(DMSO)^{2+}-H^+]^+$ at 717.16 m/z, $[MRFA+Ru(\eta^6\text{-}p\text{-cymene})^{2+}-H^+]^+$ at 758.27 m/z and $[EHSG+Ru(\eta^6\text{-}p\text{-cymene})(pta)^{2+}-H^+]^+$ at 820.25 m/z. In each spectrum 30% relative collision energy was used, the same imposed for bottom-up experiments, and the appropriate isolation width was selected in order to include the entire and characteristic isotopic pattern typical of Pt and Ru compounds. CID spectra of Fig. 8 and Fig. 9 show the stability of Pt-Met, Pt-His, Ru-Met and Ru-His bonds at the energy tested while a partial loss of Pt ligands (NH_3 and DMSO, Fig. 8b) and Ru ligands (pta and η^6-*p*-cymene, Fig. 9) is observed.

It is also interesting to notice the presence of fragments containing Ru^{4+} (attributions verified through simulations not reported here) in the CID spectrum of $[MRFA+Ru(\eta^6\text{-}p\text{-cymene})^{2+}-H^+]^+$ probably due to some rearrangements and/or redox processes during fragmentation in IT.

4. Conclusions

In this work we have designed a general and systematic protocol to test, a priori, the stability of metallodrug-protein adducts under the typical conditions of the FASP/bottom-up mass spectrometry approach. This study may turn helpful to scientists working in the field of metallomics and investigating metallodrug/protein interactions. The protocol was specifically applied to two representative model systems, Cyt *c*-CDDP and Cyt *c*-RAPTA-C. Cyt *c*-CDDP adducts were stable in all tested conditions while Cyt *c*-RAPTA-C adducts manifested a remarkable instability in the ammonium bicarbonate buffer 50 mM. This latter finding supports the need to perform a test-protocol of this kind when starting any extensive bottom-up MS investigation of protein-metallodrug systems, especially with novel, or scarcely studied, metal complexes. In fact, the nature of the metal, of its ligands and of the protein microenvironment are variables that undoubtedly may affect the stability of the metal-protein coordination bond during the bottom-up approach and are not easy to predict.

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Appendix A

Electronic supporting information.

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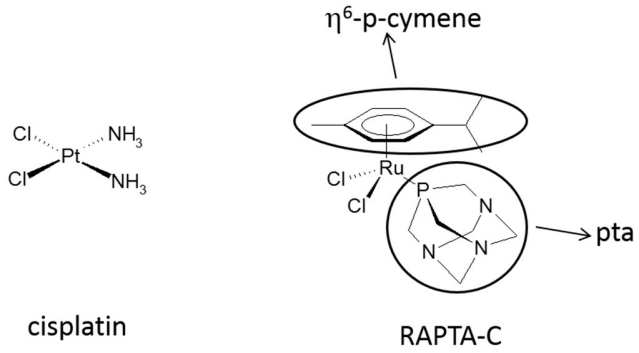


Fig. 1. Structures of the metal complexes used in this study.

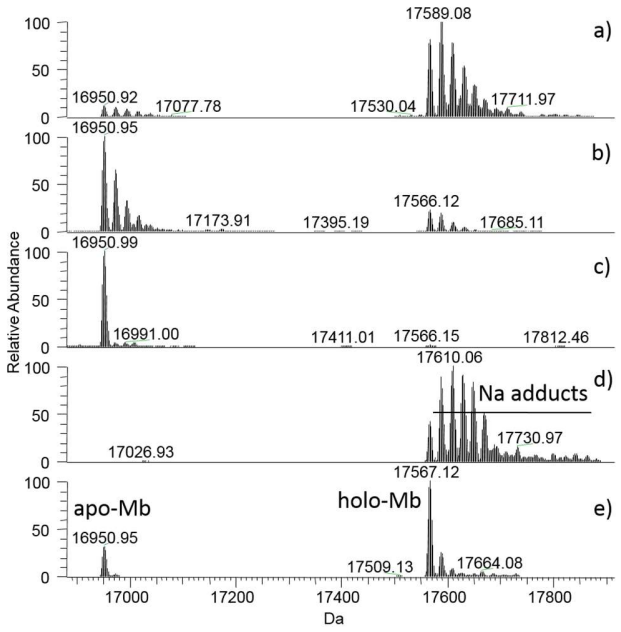
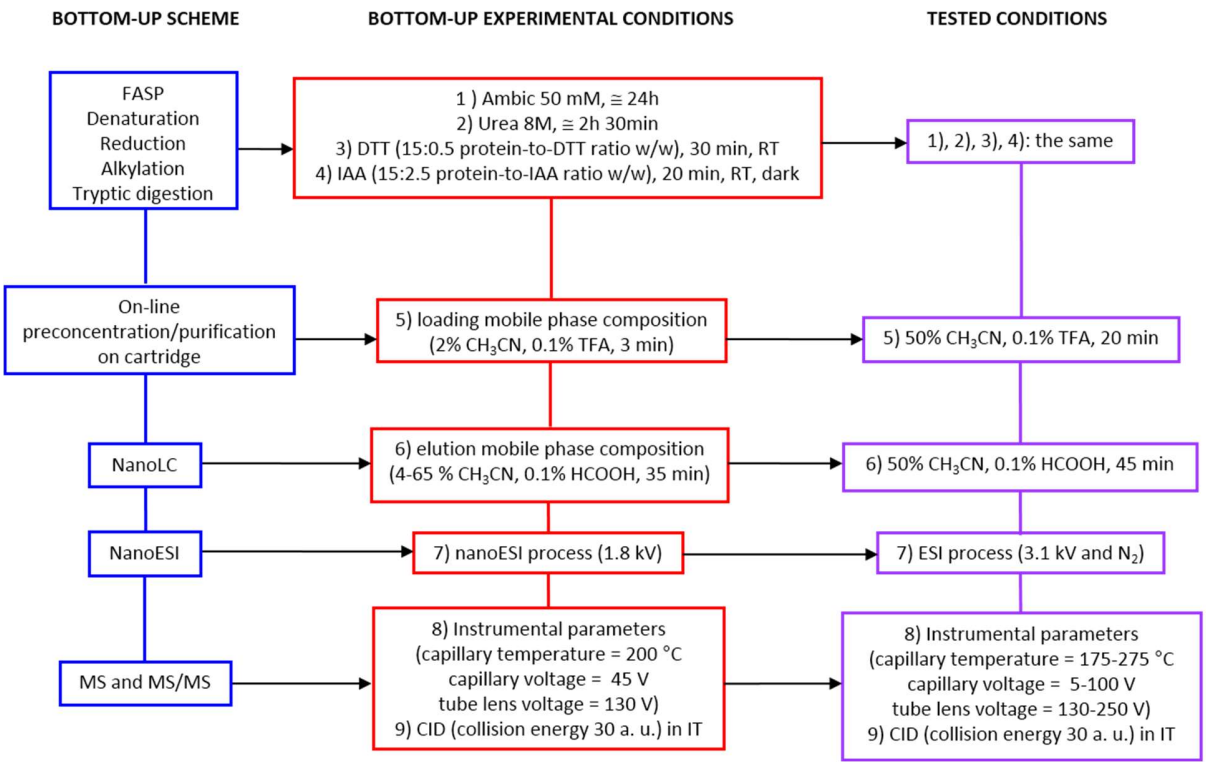


Fig. 2. ESI-Orbitrap deconvoluted mass spectra of holo-Mb solutions acquired in different preparative and instrumental conditions: a) H_2O, capillary temperature = 220 °C, tube lens voltage = 230 V; b) H_2O/CH_3CN 1/1, capillary temperature = 220 °C, tube lens voltage = 230 V; c) H_2O/CH_3CH with 0.1% HCOOH, capillary temperature = 220 °C, tube lens voltage = 230 V; d) H_2O, capillary temperature = 175 °C, tube lens voltage = 140 V; e) H_2O, capillary temperature = 275 °C, tube lens voltage = 250 V. All these spectra were acquired with capillary voltage = 45 V. The different quantity of Na adducts present in e) in comparison to a), b) and d) is due to different batches of holo-Mb solution.



Scheme 1. Bottom-up procedure we intend to use (blue rectangles), critical conditions for metal-protein coordination bond stability (red rectangles) and conditions tested in this article (violet rectangles).

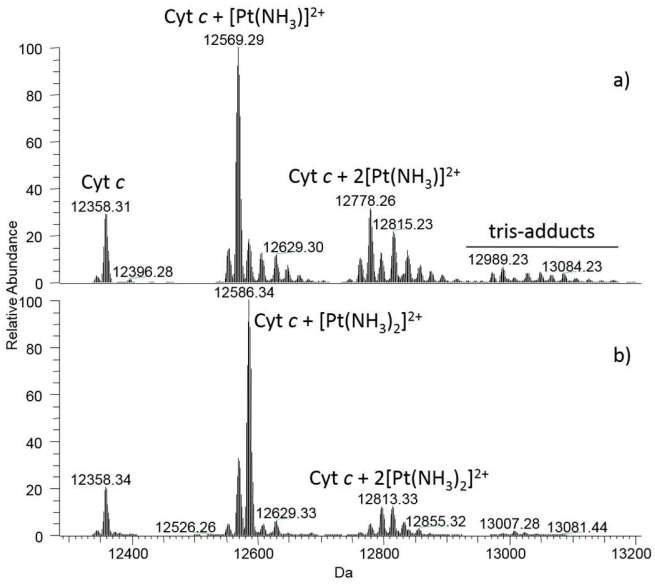


Fig. 3. ESI-Orbitrap deconvoluted mass spectra of Cyt *c*-CDDP adduct solutions after a) 144 h incubation in 20 mM AA buffer pH 6.8 and b) 144 h incubation in 20 mM AA buffer pH 6.8 and following treatment, on filter, with Ambic, urea, DTT and IAA in the times and quantities specified in the first violet rectangle of Scheme 1.

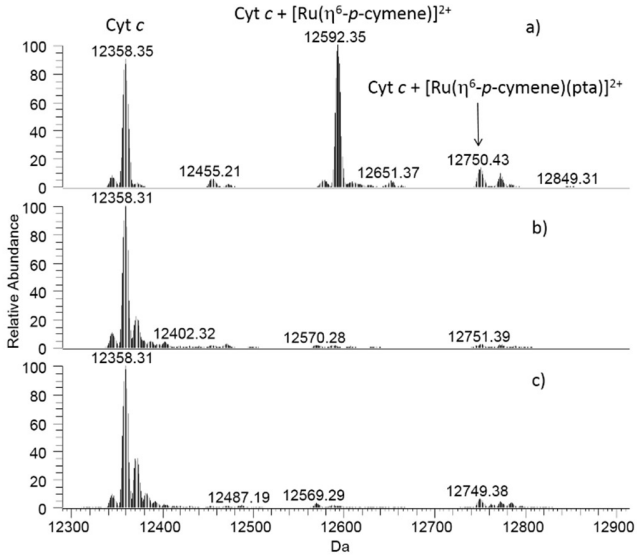


Fig. 4. ESI-Orbitrap deconvoluted mass spectra of Cyt *c*-RAPTA-C adduct solutions after a) 72 h incubation in H_2O, b) 72 h incubation in H_2O and following treatment, on filter, with Ambic, urea, DTT and IAA in the times and quantities specified in the first violet rectangle of Scheme 1, c) 72 h incubation in H_2O and following treatment, on filter, with Ambic 50 mM.

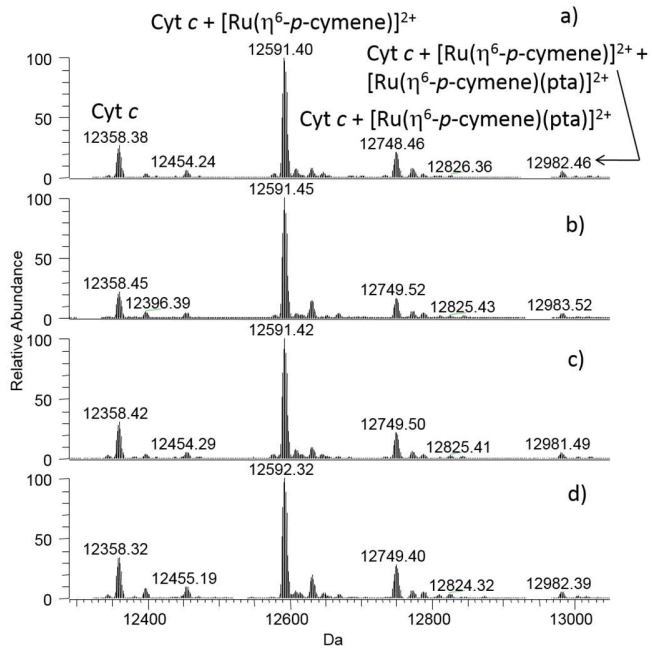


Fig. 5. ESI-Orbitrap deconvoluted mass spectra of Cyt *c*-RAPTA-C adduct solution after 72 h incubation in H_2O and different permanence time in CH_3CN/H_2O 1/1 with 0.1% HCOOH: a) t = 0, b) t = 5 min, c) t = 20 min, d) t = 45 min.

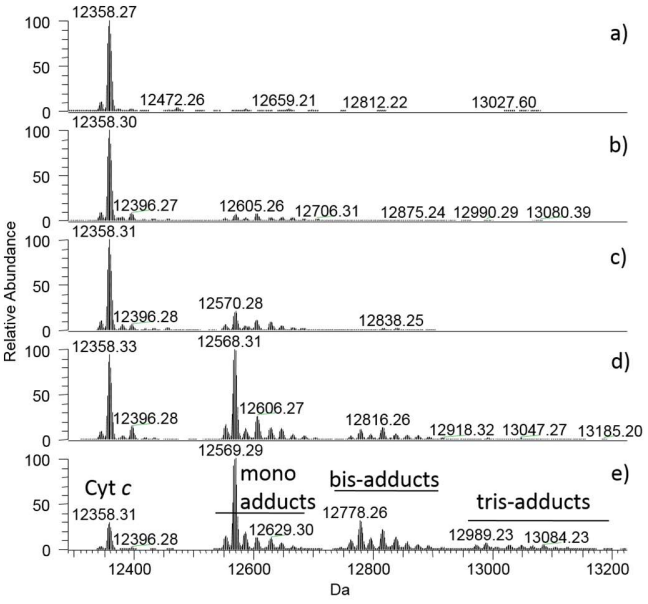


Fig. 6. ESI-Orbitrap deconvoluted mass spectra of Cyt *c*-CDDP adduct solution at different incubation times in 20 mM AA buffer pH 6.8: a) t = 0, b) t = 24 h, c) t = 48 h, d) t = 72 h, e) t = 144 h.

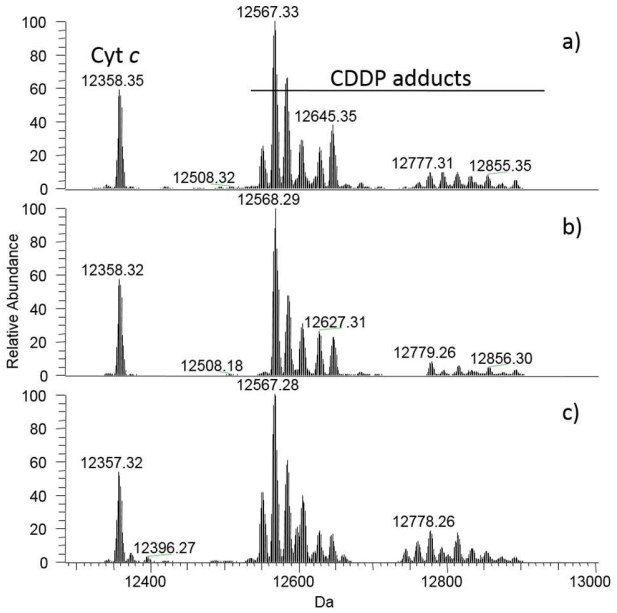


Fig. 7. ESI-Orbitrap deconvoluted mass spectra of Cyt *c*-CDDP adduct solution acquired with different instrumental conditions: a) capillary temperature = 220 °C, tube lens voltage = 230 V; b) capillary temperature = 175 °C, tube lens voltage = 130 V; c) capillary temperature = 275 °C, tube lens voltage = 250 V. All these spectra were acquired with capillary voltage = 45 V.

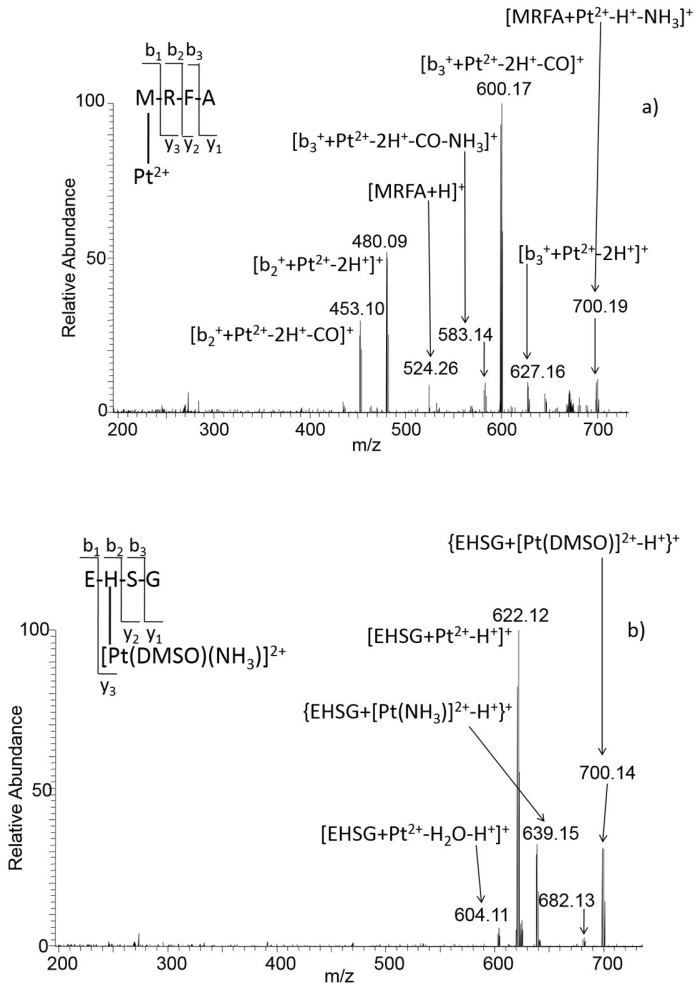


Fig. 8. ESI-CID spectra (fragmentation in IT, acquisition in Orbitrap) of a) $[MRFA+Pt^{2+}-H^+]^+$ and b) $[EHSG+Pt(NH_3)(DMSO)^{2+}-H^+]^+$. Relative collision energy = 30% for both spectra; isolation width = 4 Da in a) and 5 Da in b).

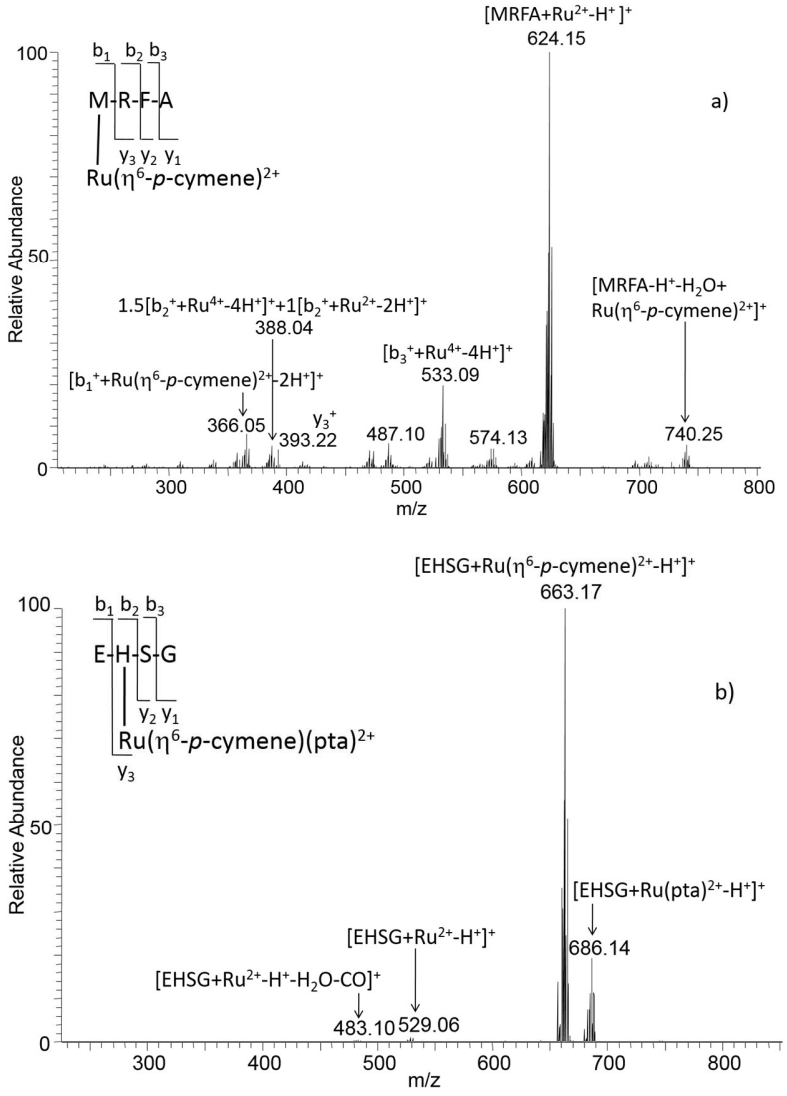


Fig. 9. ESI-CID spectra (fragmentation in IT, acquisition in Orbitrap) of a) [MRFA+Ru(η^6-*p*-cymene)$^{2+}$-H$^+$]$^+$ and b) [EHSG+Ru(*η6-p*-cymene)(pta)$^{2+}$-H$^+$]$^+$. For both spectra: relative collision energy = 30% and isolation width = 14 Da.