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Protein metalation by anticancer metal-based drugs revealed through the integration of ESI-MS and XRD measurements. State of the art and open issues

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Over the past 2 decades, solid evidence has emerged to suggest that the mode of action of certain classes of metal-based anti-cancer drugs (e.g., several ruthenium and gold compounds) is largely mediated by their interaction with cellular proteins rather than direct binding to DNA. This has sparked interest in studying the molecular mechanisms of protein metalation by anti-cancer metallodrugs in greater detail, mainly through combining ESI-MS and XRD measurements. Our integrated studies on small model proteins began in 2013, and this approach was later consolidated and summarised in two comprehensive publications in 2017, outlining the general investigative protocol, its development, and future perspectives. These studies have provided a deeper understanding of protein metalation processes at a molecular level. Based on an extensive collection of examples, we determined the main features of the metalation of several small model proteins (such as lysozyme and RNase A) by various classes of anti-cancer metallodrugs (e.g., platinum-, ruthenium- and gold-based) and identified the general rules of protein binding. Subsequently, the focus shifted towards analysing the metalation of larger proteins of great biological interest, such as human ferritin and serum albumin. Examples of these studies are provided and discussed. Finally, we offer a critical review of the issues that remain unresolved in the field of protein metalation by metal-based drugs, outlining prospects for future work.

KEYWORDS

ESI mass spectrometry, gold, protein metalation, ruthenium platinum anticancer drugs, X-ray crystallography

1 Introduction

Protein metalation is the process by which proteins acquire metals to form stable metal-protein complexes, primarily via the formation of strong coordinative bonds between the metal center and selected protein side chains (Osman and Robinson, 2023; Marzo et al., 2020). A correct metalation process is crucial for metalloproteins to achieve their proper enzymatic activity and/or 3D structure. Traditionally, the protein metalation process concerns the main physiological transition metals (e.g., iron, copper and zinc) within the context of metal homeostasis and trafficking (Peana et al., 2024). Indeed, to reconstitute functional holo-proteins, which often possess vital catalytic properties (metalloenzymes),

these metals must be properly incorporated into the 'preformed' protein binding sites (Valdez et al., 2014). The complex molecular mechanisms that govern metal transfer within cells and their accurate integration into protein binding sites have been thoroughly investigated within the broader context of metal trafficking studies (Aulakh et al., 2025). A few review articles on this topic have been published, revealing the great complexity and sophistication of the cellular machinery involved as it is the case with copper (Tsang et al., 2021). Conversely, mis-metalation—i.e., the incorporation of the "wrong" metal in a certain metalloprotein or the binding of non-physiological metals to a protein—is usually dangerous and should be avoided (Foster et al., 2022; Camponeschi and Banci, 2023). For example, the strong binding of non-physiological metals to cellular proteins, often accompanied by protein loss of function and cell damage, is the basis for the toxic effects of well-known poisonous "*heavy metals*", such as mercury, cadmium and lead (Gailer, 2007; Geri et al., 2024; Jomova et al., 2025). Yet, in some cases, the binding of an aberrant metal to a protein can turn beneficial, as it occurs with certain *metal-based drugs* and can mediate their therapeutic actions. Overall, these arguments demonstrate how the concept of protein metalation is a broad one and extends far beyond the physiology of transition metals, having a significant impact on the contiguous fields of metal toxicology and inorganic pharmacology. Here, we will focus specifically on anticancer metal-based drugs. Over the last 2 decades, the role of protein metalation in the mode of action of anticancer metallodrugs has been highlighted, thus surpassing the classical "DNA paradigm" for the mechanism of platinum drugs (Wang and Lippard, 2005; Jomova et al., 2025). Several studies on this topic have been published in the last 2 decades, aiming to characterize the interaction of metallodrugs with a variety of proteins at a molecular level (Foster et al., 2022). It is now evident that these direct interactions play a crucial role in determining the anti-cancer mechanisms and toxicity profiles of certain classes of metallodrugs (Vitali et al., 2025). The case of anticancer gold-based drugs generally acting as potent inhibitors of the selenoenzyme thioredoxin reductase (TrxR) is paradigmatic. Indeed, it has been demonstrated that gold compounds can strongly inhibit this enzyme owing to the high affinity of gold(I) species for the selenolate group in the active site (Bindoli et al., 2009; Pratesi et al., 2014), based on HSAB considerations. Even in the case of gold (III) species, it has been documented that TrxR inhibition is preceded by reduction of gold (III) to gold(I) (Bindoli et al., 2009). In turn, the strong inhibition of the enzyme causes a disruption of redox homeostasis and significant intracellular oxidative stress, eventually leading to cancer cell death.

2 A joint ESI MS and XRD investigative protocol for small model proteins

Completely characterizing the products formed in the reaction between proteins and metallodrugs can be very challenging, but it can provide valuable insight into the main features of the metal-binding process. Just 15 years ago, this kind of information was almost entirely absent from the literature. Leveraging the technical expertise and knowledge of research groups in Naples and Florence, we realized that conducting independent electrospray ionization (ESI) mass spectrometry (MS) and single crystal X-ray diffraction (XRD) studies on the same metallodrug-protein systems could provide considerable complementary information in the mode of protein metalation by various metallodrugs. This would allow us to elucidate the main characteristics of the binding process and would help to address the existing gap in literature. The systematic joint application of these two approaches to the adducts formed when metallodrugs react with selected model proteins such as lysozyme, ribonuclease A and cytochrome c proved to be a highly effective investigative strategy (see, for example, Messori et al., 2013a; Ferraro et al., 2015b; Messori et al., 2014b). These proteins were chosen because they are relatively small, soluble, stable, and commercially available at a low cost. They are also well suited to analysis by ESI MS and undergo relatively straightforward crystallization under different experimental conditions, producing crystals that are highly resistant to the soaking procedure (Russo Krauss et al., 2017). These features make them ideal for the intended investigative approach. After a long series of efforts, we were able to define a general protocol for characterizing protein metalation by metal-based drugs; the experimental protocol was rapidly standardized and published in Chem. Eur. J (Merlino et al., 2017). and Chem. Commun. (Messori and Merlino, 2017). Integrating data from ESI MS and XRD measurements typically yielded a consistent picture of the interactions between metal-containing fragments derived from the studied metal compounds and the investigated proteins (Figure 1). Applying this strategy yielded clear information on the type of adduct formed, the nature and number of protein-bound metal fragments, the location of metal-binding sites, the rate of adduct formation, and the relative stability of the adduct in a considerable number of metallodrug–protein adducts. This gave us extensive insight into the general features of the protein metalation process (Messori and Merlino, 2017; Merlino, 2021; Ferraro and Merlino, 2025). Some more recent cooperative works of our research groups further testify the effectiveness of this type of approach (Ferraro et al., 2020; 2024; 2025; Cirri et al., 2024), demonstrating its utility also in characterising metalation of nucleic acids (Tito et al., 2023; Troisi et al., 2024).

The general picture emerging from these studies, concerning the metalation of selected model proteins by the most important platinum- (Messori et al., 2014a) ruthenium- (Vergara et al., 2013; Messori and Merlino, 2014; Tamasi et al., 2017)) and gold-based drugs (Messori et al., 2013b; Russo Krauss et al., 2014), is as follows: these metallodrugs are activated by the release of at least one weak ligand or by reduction. The activated metal species then interacts with the protein surface. Metal binding occurs selectively at the level of specific protein side chains. Original metal ligands may be released and lost during the metal-binding process. However, they play a crucial role in directing the metals towards their final binding sites. Indeed, non-covalent interactions in the early stages of protein binding can significantly impact the kinetics of the process and drive product formation. Tight coordinative bonds then form gradually between the metal center and a protein side chain. The resulting "covalent" adduct may then undergo further transformations. Notably, the aforementioned metal centers (Pt, Ru and Au) exhibit remarkable selectivity for certain protein side chains (His, Met, Cys and Asp), although other side chains (Gln, Glu and Lys) may also be involved in the

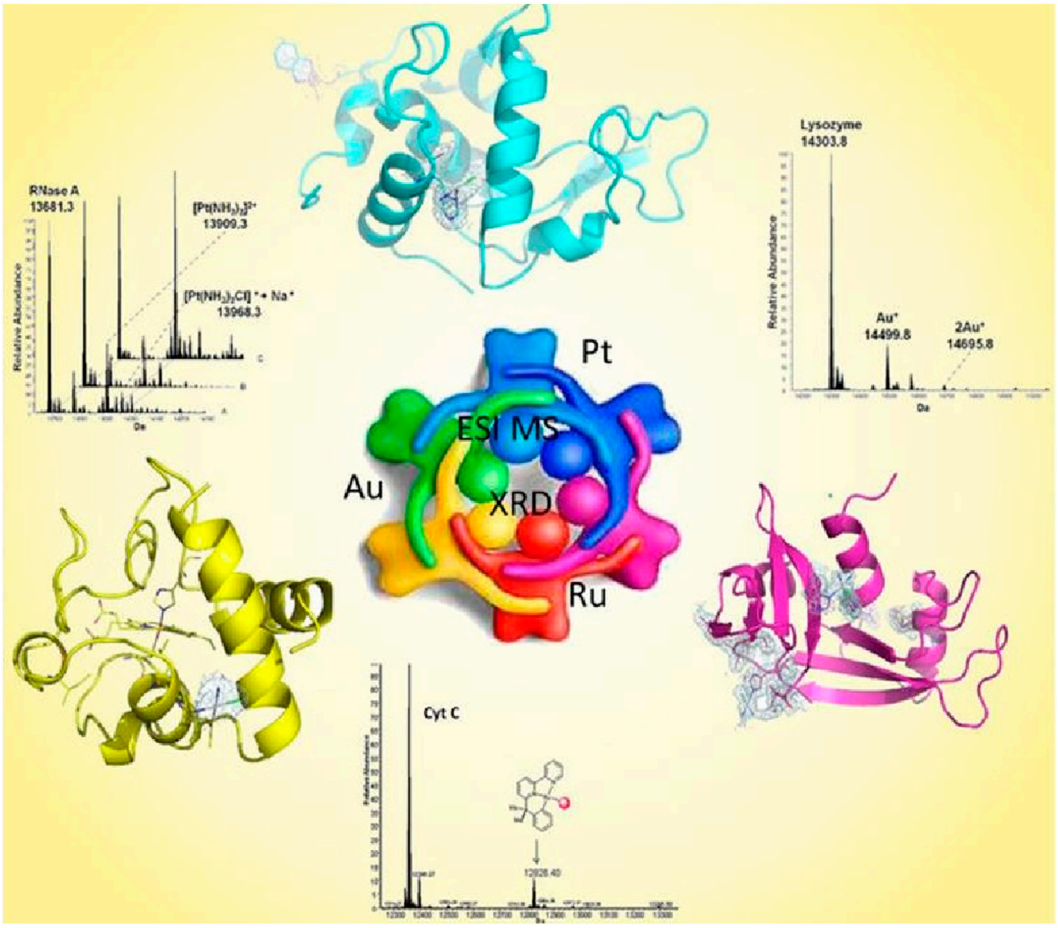

FIGURE 1
Integration of ESI-MS and XRD measurements yields a consistent picture of the interactions between metal-containing fragments and model proteins. The figure shows representative mass spectra and crystal structures of adducts formed with RNase A, lysozyme, and cytochrome c upon reaction with Pt, Au, and Ru compounds, illustrating the complementary insights provided by the combined approach. Reproduced with permission from Merlino et al., 2017.

recognition process. While the protein metalation process is primarily governed by general inorganic chemical principles (e.g., the hard and soft acid and base theory), reactivity may also be influenced by the intrinsic properties of the interacting protein side chains. These properties can be impacted by factors such as the local environment, electrostatic potential, solvent accessibility, the pKa of neighboring residues, and solution conditions.

In this frame, histidine residues offer a representative example: although chemically identical, they can exhibit markedly different reactivities depending on their local protein environment. In several protein-metallodrug adducts, metalation occurs preferentially at solvent-exposed histidines located in favorable electrostatic environments, whereas buried or hydrogen-bonded histidines remain essentially unreactive. Likewise, the selective binding of gold(I) species to specific cysteine residues in human H-chain ferritin is critically governed by local accessibility and neighboring residues. This is the case of some recent finding, where the formation of unusual multinuclear gold clusters rather than the expected mononuclear adducts was observed (Cirri et al., 2024; Cosottini et al., 2024).

3 Beyond the small model proteins: the case of the human H-chain ferritin

The investigation strategy reported above was highly effective for several small model proteins. However, we realized that it would be necessary and desirable to extend the characterization to metallodrug adducts of larger, more biologically relevant proteins. In subsequent years, we therefore attempted to analyze increasingly larger proteins, such as carbonic anhydrase, serum albumin, ferritin and transferrin, among others. Often, applying the two complementary techniques together to these systems proved problematic. Various factors can cause difficulties. The crystallisation of large proteins is similar to that of small ones. However, obtaining crystals of large proteins that are useful for X-ray diffraction analysis is complicated by sample heterogeneity, post-translational modifications and the presence of regions of the polypeptide sequence with high flexibility. Furthermore, large protein crystals often contain a high proportion of solvent, which reduces their X-ray diffraction power and resistance to X-ray exposure. These features are even more pronounced when

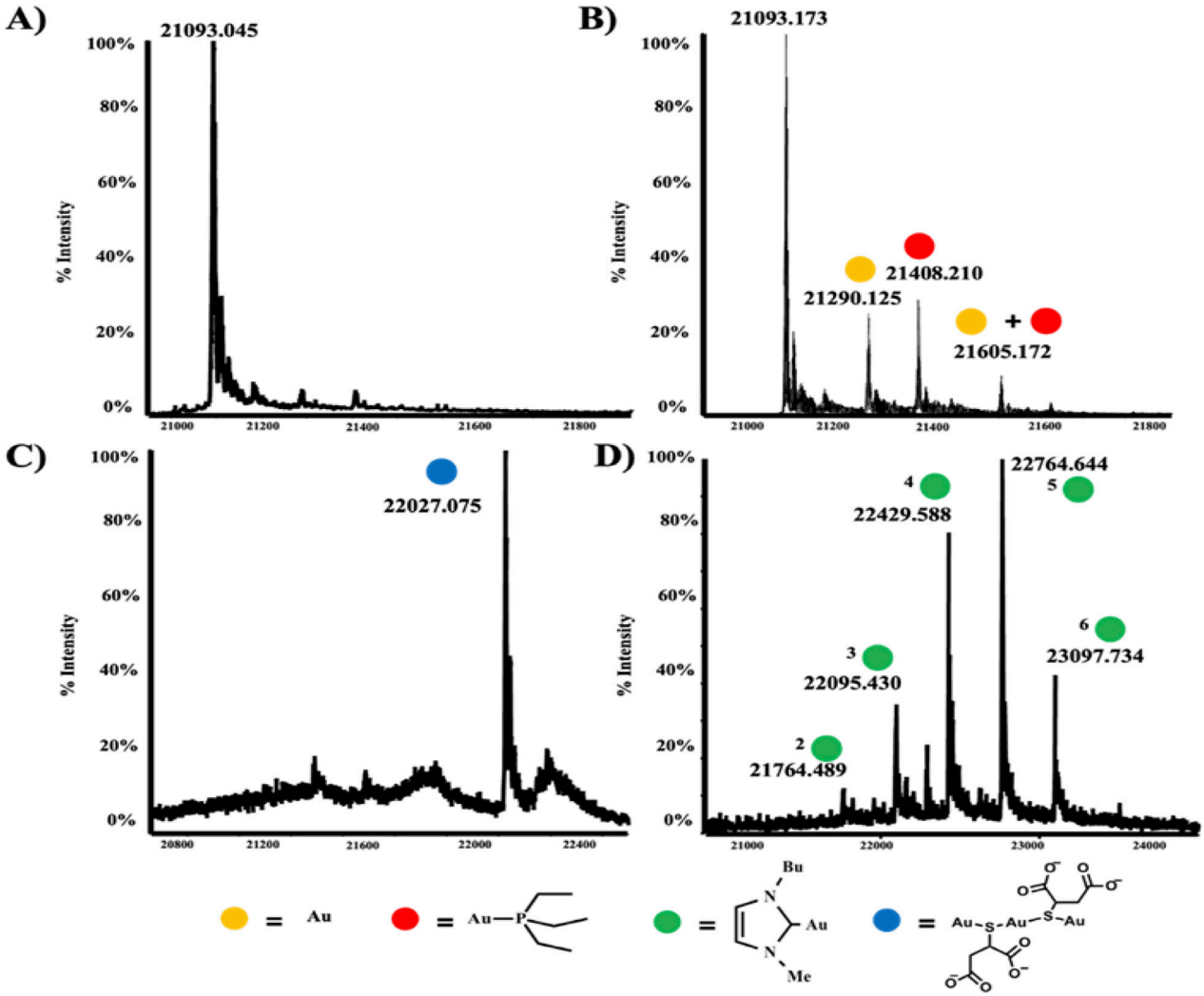

FIGURE 2
Deconvoluted ESI mass spectra of **(A)** Human H-Chain Ferritin 5 × 10^{-6} M in 20 mM Ammonium Acetate pH 6.8, incubated at 37 °C for 3 h with **(B)** Human H-Chain Ferritin 5 × 10^{-6} M with Auranofin in a 1:10 monomer-to-metal ratio **(C)** **(B)** Human H-Chain Ferritin 5 × 10^{-6} M with AuTM in a 1:30 monomer-to-metal ratio, and **(D)** Human H-Chain Ferritin 5 × 10^{-6} M with Au(NHC) in a 1:3 monomer-to-metal ratio; 1% v/v of formic acid was added just before injection. The yellow dot indicates the free Au; the red dot indicates the [AuPEt3]+ fragment; the blue dot indicates the Au3TM2Na2 species and the green dot indicates the Au(NHC) species (image courtesy of Dr. Andrea Geri).

crystals are treated with metal compounds. Acccordingly, the resulting crystallographic structures may be characterised by low intrinsic resolution, which hinders the elucidation of details of the metalation process such as the retention of original ligands.Conversely, large proteins are typically less prone to ionisation in the ESI process, so that the resulting ESI-MS spectra often have poorer resolution. Nevertheless, despite the above difficulties, brilliant results were sometimes obtained when studying the metalation of larger proteins, often relying on a single technique.

In particular, the structures of the adducts formed when cisplatin and some of its derivatives reacted with serum albumin (Ferraro et al., 2015a; 2025; Merlino, 2023) and when Pt- and Ru-based drugs react with human H-chain ferritin (Ferraro et al., 2017; Ciambellotti et al., 2018; Lucignano et al., 2022), were solved using X-ray crystallography. Cisplatin and the gold-based drug aurothiomalate were also investigated in reactions with apo- and monoferric forms of transferrin (Troisi et al., 2023; 2025a; 2025b). Finally, the structures of several metal compounds encapsulated within ferritin nanocages were reported (Ferraro et al., 2016; Pontillo et al., 2016; Monti et al., 2017; 2019). Despite the large size of these proteins, notable information on the protein metalation process was also obtained using ESI MS investigations, in the case of human serum albumin (Cirri et al., 2022; Vitali et al., 2025), ferritin (Massai et al., 2021), and carbonic anhydrase (Zoppi et al., 2020; Geri et al., 2023) for example,. To this respect the case of human H-chain ferritin is emblematic (Cosottini et al., 2023; 2024; 2025). This form of ferritin is a multimeric nanocage protein with roughly spherical architecture containing 24 identical subunits. Each subunit has a molecular weight of around 20,000, giving the protein a total molecular weight of approximately 480 kDa. The deconvoluted ESI MS spectra of human H-chain ferritin solutions are notably well

resolved, showing essentially the peak characteristic of the monomeric species. Accordingly, ESI MS analysis could be used to comparatively characterize the adducts formed in the reaction of the protein with three distinct medicinal gold compounds: auranofin, aurothiomalate (AuTM) and gold monocarbene (hereafter referred to as AuNHC). Figure 2 shows the electrospray ionization (ESI) mass spectra of human H-chain ferritin treated with an excess of each of the three gold compounds. Analysis of these spectra reveals that reaction with auranofin results in the formation of several distinct species with different stoichiometries and bound fragments. In contrast, treatment with an excess of AuTM resulted in a single species characterized by a main peak at 24,000 Da. Reaction with an excess of gold monocarbene, finally, led to a variety of adducts that differed only in the number of protein-bound Au(NHC) fragments, ranging from 1:1 to 6:1. Further ESI MS studies on three mutants lacking one or two cysteine residues revealed that gold binding is highly selective, or even exclusive, to the thiol groups of free cysteines. Interestingly, the adducts formed with AuTM or AuNHC exhibited unusual properties. In the case of AuTM, we demonstrated the formation of a small cluster containing four gold ions and three thiomalate ligands at Cys90 and Cys102. Using cryo-electron microscopy, we successfully determined the overall structure of one of the adducts formed with gold monocarbene. Solving the cryo-EM structure provided strong evidence for the formation of a four-member aurophilic cluster at Cys90 and Cys102.

4 Open issues and prospects for future work

As discussed above, combining ESI MS and XRD provides a fairly comprehensive description of the protein metalation process in single, isolated proteins. Consequently, the general features of protein metalation have been identified for a variety of metal-based drugs. In most cases, adduct formation occurs when the metal-based drug is activated, which enables the metal center to bind to a solvent-exposed side chain of the target protein. The extent of metalation depends strictly on the nature of the metal center and its specific binding preferences, as well as on the nature and relative strength of the original metal ligands. While the results obtained so far are reasonable and satisfactory, crucial issues concerning protein metalation in real cellular systems remain unanswered and require further analysis and investigation.

4.1 A deeper understanding of protein metalation in real cellular systems requires the use of sophisticated models and advanced technologies

The protein metalation studies reported above mostly refer to an idealized situation involving an extremely simplified setup, in which a single metal-based drug reacts with a single purified protein in an aqueous buffer. However, protein metalation in the cellular environment is a far more complex and unpredictable process. This is because it involves several factors, such as the presence of many low-molecular-weight components and proteins in varying amounts that compete with each other for binding to the metal center. More complex and sophisticated setups are therefore necessary, coupled to advanced technologies, as illustrated below.

4.1.1 Metalation of simple protein mixtures

One small step forward in reproducing real-life situations is analyzing the metalation of relatively simple protein mixtures. For example, in a 2009 paper (Casini et al., 2009), we attempted to simulate the cellular environment still in a very simplified manner by analyzing the metalation of an artificial protein mixture consisting of just three proteins. Further attempts in this direction are ongoing. This approach is relatively straightforward and inexpensive from a technological point of view yet can produce results that are more sophisticated than those obtained by metalating a single protein. Furthermore, the complexity of the protein mixture can be gradually increased by adding more proteins and/or small molecules, and by making appropriate choices regarding which proteins to include.

4.1.2 Metalation of the whole proteome

However, alternative approaches are needed to fully capture the true complexity of the cellular environment. To this end, the metalation of extracted proteomes or intact cells should be investigated. Specific, technology-driven approaches for this purpose have been developed in a few laboratories worldwide. Examples of these strategies, developed in the laboratories of Christian Hartinger in Auckland (Steel and Hartinger, 2020) and Hong Zhe Sun in Hong Kong (Wang et al., 2017), are based on advanced metallomics and/or metalloproteomics analytical tools, often combined with simple, smart approaches. These approaches are described in a recent review article (Wang et al., 2020), which is recommended reading for those interested in this topic.

4.2 A deeper appreciation of the biological consequences of protein metalation is needed

In our opinion, another issue that deserves much attention and further study is developing a more comprehensive understanding of the biological consequences of protein metalation. In particular, we must identify which metalation events have a significant impact on cell function and which other have little to no effect. This could be achieved by using omics technologies such as proteomics and metabolomics to investigate the impact of protein metalation on the cell as a whole. Indeed, proteomic studies followed by bioinformatic analysis can reveal whether a certain degree of protein metalation produces irreversible cellular damage that leads to cell death. Pathway analysis could then reveal which metabolic or signaling pathways are most affected, suggesting which proteins may be the effective targets whose metalation results in significant cellular damage. These findings could then be further corroborated by metabolomics studies highlighting changes in the composition of the metabolome in treated cells.

In this context, structural information obtained from ESI-MS and XRD studies can play a key role in interpreting and validating proteomic and metabolomic data. Detailed knowledge of preferred metal-binding residues, coordination geometries and site-specific solvent accessibility enables assessment of whether proteins

identified in metalloproteomic analyses represent chemically plausible targets. Furthermore, structural insights help to distinguish stable, functionally relevant metalation events from transient or non-specific interactions, thereby supporting the biological interpretation of omics-derived datasets.

5 Discussion

We have shown here how protein metalation is a central concept to understand the molecular basis of the biological actions of metal-based drugs. This implies that appropriate experimental strategies are required to elucidate the process of protein metalation in structural and functional detail. Recently, we developed a systematic joint approach to characterize protein metalation in isolated model proteins, combining X-ray diffraction and ESI MS. This approach was fairly successful, unveiling the structural basis of protein metalation in simple model proteins at the atomic level. However, other aspects of the process, particularly the dynamic ones concerning the formation and evolution of the adducts and the possible transfer of protein-bound metals among the potential binding sites, both intraprotein and interprotein, are still under investigation. Further progress can be achieved even with the support of computational methods.

These approaches indeed constitute powerful complements to experimental investigations. MD simulations can shed light on protein flexibility, transient metal-protein interactions, and the dynamic accessibility of potential binding sites preceding stable coordination. In parallel, DFT calculations allow detailed analysis of the electronic structure of metal centers, estimation of binding energies, and rationalization of ligand exchange and redox processes observed experimentally. Together, these computational strategies help to bridge the gap between static structural information and the dynamic evolution of metallodrug-protein adducts.

As our initial approach was limited to studying small, isolated model proteins, we realized that we needed to extend our research to larger proteins of greater biological significance and to real cellular systems, taking into account their inherent complexity. In other words, the protein metalation scheme developed using simple models must be adapted to address the complexity of real proteomes in living systems. Notably, thanks to the research activities of a few leading laboratories worldwide, advanced methodologies are being developed to reveal the metalation of cellular proteomes in real biological systems. This will enable us to define the actual patterns of protein metalation inside cells and establish which proteins are metalated and to what degree. Complementary omics studies will then reveal the functional consequences of such protein metalation, in terms of alterations to cell homeostasis and selective perturbation of the various signaling and metabolic pathways. Integrating all these results will enable us to retrospectively assess whether and to which extent the metalation processes observed in single, isolated model proteins are representative of the global cellular effects of metallodrug treatment. This perspective article aims to provide an overview of studies in the field of protein metalation since 2017 (Merlino et al., 2017; Messori and Merlino, 2017), and suggests future scenarios and lines of investigation in response to new and more ambitious challenges.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

AM: Conceptualization, Writing – review and editing, Writing – original draft, Funding acquisition, Resources. TM: Visualization, Resources, Software, Conceptualization, Writing – review and editing. LM: Resources, Investigation, Writing – review and editing, Writing – original draft, Funding acquisition, Conceptualization.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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