Chem Soc Rev

RSC Publishing

REVIEW ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Chem. Soc. Rev.*, 2013, **42**, 6186

Application of mass spectrometric techniques to delineate the modes-of-action of anticancer metallodrugs

Christian G. Hartinger,*[abcd] Michael Groessl,[e] Samuel M. Meier,[bc] Angela Casini[f] and Paul J. Dyson[d]

Received 31st December 2012

DOI: 10.1039/c3cs35532b

www.rsc.org/csr

Mass spectrometry (MS) has emerged as an important tool for studying anticancer metallodrugs in complex biological samples and for characterising their interactions with biomolecules and potential targets on a molecular level. The exact modes-of-action of these coordination compounds and especially of next generation drug candidates have not been fully elucidated. Due to the fact that DNA is considered a crucial target for platinum chemotherapeutics, metallodrug–DNA binding studies dominated the field for a long time. However, more recently, alternative targets were considered, including enzymes and proteins that may play a role in the overall pharmacological and toxicological profile of metallodrugs. This review focuses on MS-based techniques for studying anticancer metallodrugs *in vivo*, *in vitro* and *in situ* to delineate their modes-of-action.

[a] *School of Chemical Sciences, The University of Auckland, 1142 Auckland, New Zealand. E-mail: c.hartinger@auckland.ac.nz; Web: http://hartinger.wordpress.fos.auckland.ac.nz/; Fax: +64-9-373-7422; Tel: +64-9-373-7599 ext 83220*

[b] *University of Vienna, Institute of Inorganic Chemistry, Währinger Str. 42, A-1090 Vienna, Austria*

[c] *Research Platform "Translational Cancer Therapy Research", University of Vienna, Institute of Inorganic Chemistry, A-1090 Vienna, Austria*

[d] *Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland. E-mail: paul.dyson@epfl.ch; Fax: +41-21-69-39885; Tel: +41-21-69-39854*

[e] *University of Vienna, Institute of Analytical Chemistry, Währinger Str. 38, A-1090 Vienna, Austria*

[f] *Pharmacokinetics, Toxicology and Targeting, Research Institute of Pharmacy, University of Groningen, 9713 AV Groningen, The Netherlands*

1 Introduction

Bioinorganic chemistry is a scientific discipline at the intersection of inorganic chemistry, biology, biochemistry, pharmacy and medicine.[1] At first sight it may appear surprising that inorganic chemistry plays a central role in living organisms. Indeed, many essential enzymes and proteins contain metals at their active sites with crucial catalytic and structural roles, and the potential of metals as pharmaceuticals was even identified by various ancient civilisations.

Early in the last century, the 1908 Nobel laureate in Physiology or Medicine, Paul Ehrlich, discovered salvarsan, an arsenic-based

Christian G. Hartinger

Christian G. Hartinger received his PhD in Chemistry in 2001 from the University of Vienna under the supervision of Prof. Bernhard K. Keppler. From 2001 he worked as a research assistant at the same department until he joined the group of Paul Dyson at the EPFL as a Schrödinger Fellow in 2006. Upon his return to the University of Vienna in 2009, he obtained his habilitation in Inorganic Chemistry and was appointed Associate Professor at the University of Auckland in 2011.

Michael Groessl

Michael Groessl completed his PhD studies in Chemistry at the University of Vienna supervised by Bernhard K. Keppler in 2008 and subsequently joined the group of Paul Dyson at the EPFL as a Schrödinger Fellow. From 2011 to 2012, he worked at the Max-Planck-Institute of Molecular Cell Biology and Genetics (MPI-CBG) in the Shevchenko lab. Currently, he is employed at the Institute of Analytical Chemistry at the University of Vienna. His research interests focus on proteomics and metallomics studied by mass spectrometry and hyphenated techniques.

syphilis therapeutic. In more recent times, cisplatin, a platinum-containing anticancer agent, also considered as a landmark discovery in metallodrug research, was approved for worldwide clinical use in 1978 and is nowadays included in approximately 50–70% of therapeutic schemes in the treatment of cancer together with the 2nd and 3rd generation compounds carboplatin and oxaliplatin (Fig. 1). Pt-based anticancer agents have reached "blockbuster" status in the pharmaceutical industry with turnovers of over 1 billion $ per year.[2,3] DNA was identified early as the primary target of Pt(II) anticancer agents: adduct formation causes changes in DNA structure, hindering replication and transcription, which ultimately results in the induction of apoptosis, *i.e.* programmed cell death.[4] Chemotherapy with platinum drugs is often accompanied by serious side effects, but is also limited to a relatively small number of curable tumours and especially intrinsic and acquired resistance hampers the therapeutic efficacy. During the last few decades, various strategies were investigated for overcoming these problems, *e.g.* ligand modification, development of polynuclear systems, changing the oxidation state and coordination geometry, using alternative metal centres and applying drug delivery/targeting protocols.[3,5]

These efforts resulted in the development and clinical testing of ruthenium, gallium and titanium complexes[6] in addition to more than 30 Pt compounds. Ruthenium compounds such as the octahedral indazole complex KP1019, which shows activity against colorectal tumours or the imidazole–DMSO compound NAMI-A, exhibiting antimetastatic activity, were shown to be less toxic than platinum complexes in humans. This is thought to be in part due to a higher degree of selectivity by potentially exploiting serum protein-mediated transport into the tumour and activation-by-reduction therein. KP1019 and NAMI-A are currently undergoing clinical trials and are promising candidates for non-platinum anticancer chemotherapeutics.[3] In contrast, Ga compounds, such as the quinolinolato containing compound KP46, are thought to follow a different mode of action *via* interference with the iron metabolism. Notably, inhibition of the enzyme ribonucleotide reductase involved in DNA synthesis appears to be responsible for its antineoplastic activity. KP46 and tris(maltolato)gallium entered clinical trials in the early 2000s.[3]

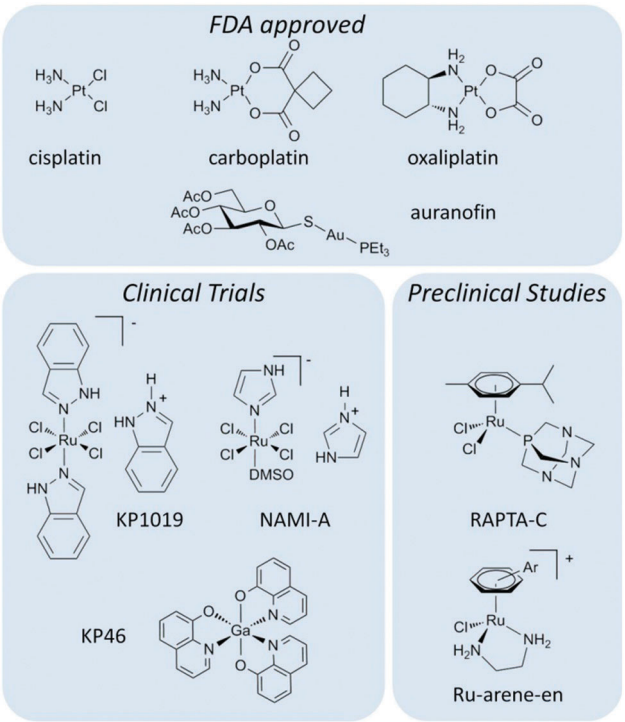


Fig. 1 Chemical structures of representative metal-based drugs and drug candidates.

Samuel M. Meier

Samuel M. Meier studied at the EPFL, the University of Edinburgh and the ETH Zurich, where he obtained his master's degree in Chemistry. He has received his PhD under the supervision of Bernhard K. Keppler and Christian G. Hartinger at the University of Vienna in 2013.

Angela Casini

Angela Casini completed her PhD in Chemistry at the University of Florence in 2004 and was a senior scientist at the EPFL funded by the Swiss National Science Foundation (2008–2012). She is currently assistant professor at the University of Groningen, funded by a Rosalind Franklin Fellowship, where she studies the molecular mechanisms of anticancer metallodrugs.

Paul J. Dyson

Paul J. Dyson received his PhD at the University of Edinburgh. He moved to Imperial College as a fixed-term lecturer and in 1995 obtained a Royal Society University Research Fellowship which he held at Imperial College and subsequently the University of York. He joined the EPFL in 2002 and is currently the director of the Institute of Chemical Sciences and Engineering.

Besides the abovementioned coordination compounds, several classes of other metal complexes and organometallic compounds, including Ag, Au, Fe, Gd, Rh, Ru(II)–arene and Ti compounds, exhibit promising anticancer, malarial, fungal, microbial and viral activity at least in preclinical studies, and Ga, Gd, Re and Tc compounds are in addition used for imaging purposes.[6,7]

Organometallic Ru(II)–arene anticancer agents are of particular interest due to their potential for systematic modulation for specific targets, *i.e.* the Ru–arene interaction is usually stable in a biological environment unlike the ligands of many coordination complexes.[8,9] Moreover, like Pt(II) anticancer agents they often exert their biological effect by coordinative binding to a target *via* the hydrolysis of Ru–chlorido bonds with the formation of reactive aqua species. Among the best (preclinically) developed organometallic drug candidates are the Ru(II)-based RAPTA (Fig. 1) compounds for treatment of cancer and, notably, DNA is not thought to be the primary target.[10] In contrast, Ru(II)–arene species bearing ethylenediamine ligands (Fig. 1) seem to target DNA and exhibit potent activity *in vitro* and against *in vivo* models. Fe in the form of ferrocene derivatives of tamoxifen and Au compounds constitute two further classes of metallodrugs with potential as anticancer chemotherapeutics.[6]

Chemotherapy in general and with Pt compounds in particular is associated with severe side effects such as nephro-, hepato-, oto-, neurotoxicity, nausea and hair loss and the therapeutic effect is limited to a small number of tumour types.[11] The mechanisms of action of metal drugs are complicated because of their reactivity to nucleophilic DNA and protein constituents and are therefore not fully known yet. It is likely that it involves DNA and protein binding and mass spectrometric (MS) methods can be helpful in improving our understanding of their biological action.

Since the invention of the first mass spectrometers by Thomson in 1912, MS has evolved into an indispensable analytical tool covering many different applications in synthetic, environmental and medicinal chemistry as well as molecular biology and life sciences amongst others.[12] MS relies on the detection of positive or negative ions, which are separated in the gas phase according to their mass-to-charge (m/z) ratio. Ionisation, ion filtering/mass analysis and detection (see in the next subsections for important examples of mass spectrometer components) are therefore crucial steps in MS experiments and different ionisation techniques and mass analysers are available for this purpose,[13] each with their characteristic fields of application and own merits, in particular with respect to metal-based drugs. For example, inductively-coupled plasma (ICP)-MS relies on the atomisation and ionisation of the sample in the ICP, thus generating singly-charged positive ions which can be detected in the MS.[14] Consequently, ICP-MS tends to be employed for the determination of total metal contents in biological samples or hyphenated to separation methods for speciation. In contrast, soft ionisation techniques such as electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) allow the analysis of intact biomolecules, a discovery for which the 2002 Nobel Prize in chemistry was awarded. ESI-MS is particularly widely used to analyse interactions between metals and biomolecules at the molecular level and to identify biological targets. Most mass analysers are limited in their mass range; consequently biomolecules with high molecular weights require multiple charges on an analyte ion, thereby shifting the m/z ratios of the target analytes into the available mass range. Due to their structural features, proteins are usually positively ionised (caused by protonation of basic amino acids), whereas DNA is most frequently transferred into the gas phase as negatively charged ions (due to deprotonation of the phosphate backbone). The fact that most medicinally relevant metals do not occur naturally in living organisms facilitates the analysis of metal-based drugs by MS in biological samples. Additionally, metal-containing compounds often show characteristic isotopic distributions which facilitate their identification. Interactions between metallodrugs and biomolecules can readily be identified by a specific increase in mass corresponding to the nature of the formed adduct.

2 Mass spectrometric instrumentation for studying metallodrugs in biological settings

2.1 Ion sources

The most common ionisation techniques for studying the interactions of metal complexes with biological molecules are ESI, MALDI and ICP (see Table 1 for a comparison of selected ion sources and mass analysers). The different ionisation methods yield highly characteristic mass spectra:

- ESI generates singly to multiply charged ions by adduct formation with protons, alkali metal ions or by deprotonation, resulting in a characteristic charge envelope for peptides, proteins and oligonucleotides.
- MALDI yields mostly singly charged pseudomolecular ions of *e.g.* protein metallodrug adducts.
- ICP produces mostly singly charged ions due to atomisation and ionisation in the plasma. Polyatomic interferences with identical m/z as the analyte ions may hamper the analysis. ICP-MS is often applied for quantification of metals in biological tissue samples but does not give structural information as do ESI and MALDI.

In cases where only very limited sample amounts are available, nano-electrospray ionisation (nESI) provides increased sensitivity. In particular, when coupled to nano-liquid chromatography (nanoLC) the method is invaluable in bioanalytical and proteomics research.[16,17]

2.2 Mass analysers

Many different mass analysers are used in MS instruments (for details on the physical principles see for example ref. 13). The quadrupole mass analyser was most commonly used in MS to separate ions of different m/z ratios on the basis of stability of their trajectories in oscillating electric fields. While it is still widespread in electron ionisation (EI)-MS and in ICP-MS, it has been replaced by linear and 3D ion traps (ITs) in combination with ESI sources in low cost and low resolution ESI instruments. More recently, high resolution mass analysers such as quadrupole-time of flight (QToF) and Fourier transform (FT)-based orbitrap have emerged in addition to FT-ion cyclotron resonance (ICR) MS

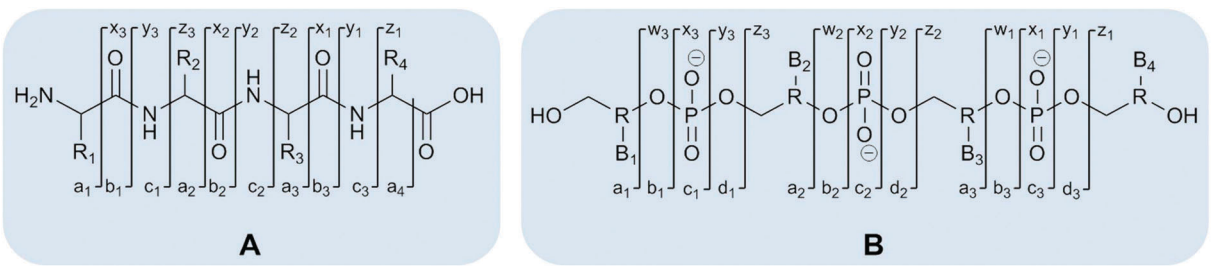
Table 1 Assessment of strengths and weaknesses of selected ion sources and mass analysers for characterisation of metal compounds in biologically relevant settings. Adapted from ref. 15–17

		Advantages	Disadvantages	Relevance to metallodrug research
Ion sources	ESI	Soft ionisation; single to multiple charges; analysis of covalent and non-covalent interactions; ease of hyphenation to CE, LC	Matrix sensitive; ion suppression; quantification	+++
	MALDI	Soft ionisation; single charges	Offline hyphenation to separation systems; quantification	++
	ICP	Element specific information; sensitivity; quantification; ease of hyphenation to CE, LA, LC	No structural data	++
Mass analysers	Triple quadrupole	Quantification; MS^2 properties	Low mass range	++
	Ion trap	Sensitivity; MS^n properties	Low mass range; quantification	+++
	ToF	Wide mass range; high resolution; high frequency of spectra recording	Limited to MS^2	++
	FT-ICR	Mass accuracy; high resolution; MS^n properties	Low mass range; costs; maintenance	++
	FT-orbitrap	High resolution; mass accuracy; MS^n properties	Low mass range; costs	+++

and represent the state-of-the-art instruments. The choice of mass analyser often depends on the application and required resolution, but commonly also on the cost. While triple quadrupole (QqQ) instruments are relatively cheap, they are normally used for quantification in metabolomics and targeted proteomics approaches. The analysis of high molecular weight samples is mainly performed with more expensive QToFs or FT-based orbitraps and ICRs, which are also used in proteomics and other applications requiring isotopic resolution and high mass accuracy (<5 ppm mass error). It must be noted that the mass resolution of QToFs is rather constant over the whole *m*/*z* range, while it decays with increasing *m*/*z*-ratios using FT-based mass spectrometers.

2.3 Fragmentation methods

Most of the mass spectrometers available offer one or more fragmentation methods which allows collecting further information about the structure and composition of the ion of interest. This is of high relevance for protein sequencing, *i.e.*, determining the order of amino acids in unknown proteins. In metallodrug research fragmentation methods are of relevance for the characterisation of metal binding sites on proteins or DNA fragments.[18–21] Collision induced dissociation (CID) is the standard fragmentation technique that is available in most commercial mass spectrometers, as is in-source decay. More recently, especially with the introduction of FT-based orbitrap- and ICR-MS, a number of additional fragmentation techniques have been introduced. These comprise infra-red multiphoton dissociation (IRMPD), higher energy C-trap dissociation (HCD), electron transfer dissociation (ETD) and electron capture dissociation (ECD). CID, IRMPD and HCD mainly give rise to b/y ions in protein samples, whereas ECD and ETD yield predominately yield c/z ions (see Scheme 1 for the nomenclature of peptide/protein and DNA fragments). Due to the underlying differences in the fragmentation mechanism, the techniques yielding b/y or c/z peptide ions can give complementary information and thereby greatly improve sequence coverage and specificity.[18] When fragmenting DNA, CID and IRMPD mainly yield a/w ions usually accompanied by base loss. ECD leads to a/w and d/z ions (Scheme 1), while ETD often results in charge reduction without fragmentation.[22] Furthermore, there are two approaches that can be pursued in designing a fragmentation experiment, originating from proteomics: top-down describes the fragmentation of intact analytes while bottom-up involves enzymatic digestion of the analyte prior to fragmentation. The advantage of bottom-up is that large analytes and highly complex samples can be analysed but for metallodrug–biomolecule-adducts, artefacts (*e.g.* coordination of the metal to another species during digestion or separation) may be generated. In top-down MS, information on the protein-bound species is



Scheme 1 (A) MS fragmentation nomenclature of the protein/peptide backbone. Fragments a_n–c_n are obtained with an intact N-terminus and x_n–z_n with an intact C-terminus. R_{1-4} denote the amino acid side chains. (B) MS fragmentation nomenclature of oligonucleotides. The fragments a_n–d_n are obtained with an intact 5′ terminus and fragments w_n–z_n with an intact 3′ terminus. R and B_{1-4} denote ribose/deoxyribose and nucleobases, respectively.

obtained directly but the approach is technically demanding and limited to low-complexity samples.[23] Both strategies can be used for the determination of metal binding sites, although the top-down approach proved to be more efficient for detecting labile metallation sites.

2.4 Hyphenation techniques

LC coupled to a mass spectrometer (LC-MS) is one of the standard analysis techniques in bioanalytical chemistry. Equipped with chromatographic columns operating on different separation principles, an enormous number of analytes can be separated, identified and quantified in a wide variety of samples and in very short time frames. Other separation techniques of relevance in this area are capillary (CE) and gel electrophoresis (GE), which can be hyphenated online and/or offline to mass spectrometers. A more recently introduced technique to the area of metallodrug research represents laser ablation (LA) hyphenated to ICP-MS. In general, the appropriate instrumental setup is chosen based on the analyte, the separation problem, the information required but also on the system complexity.[15]

2.5 Data analysis and spectra interpretation

Software applications are becoming increasingly important in the analysis and processing of MS data sets since the amount of data obtained in just a few MS runs can easily reach the gigabyte range. Software is normally supplied by the instrument manufacturers, but can also be obtained from 3rd party and open-source programmers. In particular, protein database search engines, data deconvolution (for an example see the recorded and charge-deconvoluted spectra of ubiquitin in Fig. 5), export of spectral features, quantification and more recently automatic structure assignment are some of the most useful features, however, require sufficient computing power.

3 Mass spectrometric analysis of anticancer metallodrugs: from inter-tissue distribution to molecular targets

Monitoring metal-based drugs and their metabolites in biological systems is important in order to understand their pharmacokinetics, pharmacodynamics and toxicological effects. DNA damage is considered to be the ultimate event at the basis of the therapeutic effect of classical Pt(II)-based anticancer agents, whereas for the new generation anticancer metal complexes interactions with other biomolecules such as proteins are believed to play a major role.[3] Importantly, off-target interactions with biomolecules might account for the side effects of metallodrugs that require a molecular understanding for drug optimisation. MS can provide insights into these types of questions and can be employed at different levels of biological systems for a better understanding of *in vivo* drug distribution and drug–biomolecule interactions (Fig. 2).

The distribution and toxicology of metallodrugs in living organisms, their intracellular levels and distribution, and molecular reactivity toward biomolecules as well as potential intracellular metal binding and speciation are crucial issues for drug efficacy and tolerance. Moreover, the cellular response to anticancer metallodrug exposure is a related topic of investigation in delineating the mode of action of metal-based chemotherapeutics.[24,25] The integrated evaluation of data from different experimental approaches, including bioanalytical, biophysical, biochemical and biological studies, not only provides information on how metallodrugs exert their effect but also provides valuable

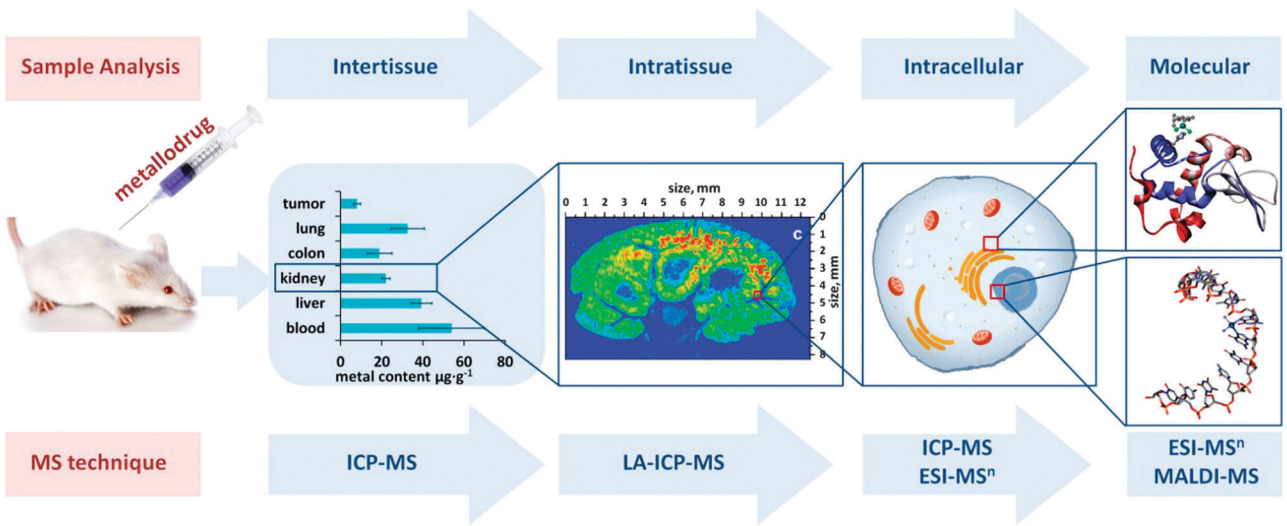


Fig. 2 Main fields of application of MS-based techniques for determining metallodrug modes-of-action: from inter-tissue distribution to molecular characterisation of metallodrug–biomolecule adducts. MS can be used in combination with various on- and offline chromatographic or electrophoretic techniques for this purpose. The metallodrug–protein (1T3P) and DNA (3LPV) crystal structures were adapted from the protein data bank. The LA-ICP-MS picture was adapted from ref. 30 © 2011 American Chemical Society.

information applied to the development of new compounds with improved pharmacological properties.

When analysing metallodrug–biomolecule interactions, the fundamentals of coordination chemistry should always be kept in mind as most metal-based anticancer agents exhibit their therapeutic effect by forming coordinative bonds with biomolecular targets although other modes of action are also known. The binding behaviour of metallodrugs is governed by the principle of hard and soft acids and bases (HSAB). A basic knowledge of coordination chemistry must be applied, but the binding is also affected by the microenvironment of each individual binding site, which is not always easy to predict, and renders a targeted design of metal therapeutics difficult.

3.1 Analysis of complex biological systems

Drug discovery has been facilitated by the fast evolution of proteomic techniques. Hyphenated chromatography or gel-based multidimensional separation techniques offer powerful and rapid means of determining the protein compositions of organisms with known genome sequences.[26] Besides proteomics, the development of metallomic approaches (study of metal species within biological systems including their interactions with biomolecules) has contributed significantly to the better understanding of anticancer metallodrugs in organisms. Many recent data have been summarised in a more specialised review,[27] and are not covered in detail in this paper.

ICP-MS has played a key role in the development of metallomics achieving high element specificity and sensitivity, especially when hyphenated to chromatographic or electrophoretic separation systems.[28] ICP-MS is commonly used for determination of metallodrug pharmacokinetics, *i.e.* determination of mechanisms of metallodrug absorption and distribution, metabolisation and excretion, often in mouse organs, blood or in cells.[29] For example, the total content of carboplatin was determined in the serum, lung and tumour in a rat model by ICP-MS and it was demonstrated that chemically-induced embolisation (emboli formation is induced to clog blood vessels) drastically increases Pt tumour accumulation. As for any analytical technique the validation of ICP-MS methods for the analysis of biological samples is crucial with regard to assessment of the method's robustness and performance by determining the limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, *etc.*[29]

Hyphenation of ICP-MS to laser ablation is of interest for mapping the spatial metallodrug intra-tissue distribution with toxicological relevance, *e.g.* the profiling of *in vivo* nephrotoxicity upon cisplatin administration.[30] However, the lack of a reliable and robust quantification method allows only semi-quantification of the intra-tissue metal content.[31] More recently, MALDI-TOF imaging was applied to study the spatial distribution of oxaliplatin in tissue sections of rat kidneys. The obtained results showed that oxaliplatin was localised at the periphery of the kidney, suggesting that the drug cannot penetrate deeply into the organ, thereby explaining its reduced nephrotoxicity in comparison to cisplatin.[32]

Cellular targets of metallodrugs include DNA, proteins and/or membrane constituents. Due to the aforementioned property of Pt(II) anticancer agents to exert their therapeutic effect by binding to DNA, analysis of the DNA isolated from the tumours of patients undergoing platinum chemotherapy, from animal studies or from *in vitro* cell culture experiments is of high interest. Again, most studies focus on the determination of total Pt content in extracted DNA samples rather than the elucidation of the structure of adducts formed and consequently ICP-MS with its unrivalled sensitivity is used for this purpose.[29] Such studies showed that upon treatment with cisplatin, only ~1% of the intracellular platinum is attached to DNA, while the fate of the remaining 99% remains largely unexplored. However, it has been demonstrated that LC-ESI-MS^2 can be used for the detection and quantification of deoxyguanosine–deoxyguanosine (GG) and deoxyadenosine–deoxyguanosine (AG) intrastrand cross-links in DNA extracted from mouse tissue samples caused by oxaliplatin binding.[33] For this purpose, three different enzymes were used to digest the extracted DNA in order to obtain simple Pt–GG and Pt–AG adducts, which were then analysed by MS^2. Remarkably, an LOD of 23 and 19 adducts per 10^8 nucleotides was obtained for Pt–GG and Pt–AG adducts, respectively.

A few studies concerning the intracellular fate of metallodrugs have been reported, *e.g.* comparison of KP1019, NAMI-A and cisplatin in a series of different cancer cell lines in order to learn more about their different therapeutic effects.[34] Notably, these studies demonstrated that the targets of the Ru-containing KP1019 are found in the cytosol rather than in the nucleus and give a clear indication for a distinct mode of action as compared to Pt(II) anticancer agents. Size exclusion chromatography (SEC)-ICP-MS revealed initial binding of KP1019 to high molecular weight proteins or protein aggregates in the cytosol, followed by redistribution to proteins with molecular weights <40 kDa after 24 h. However, the eventual targets were not established. ICP-MS-based methods do not allow molecular or structural information to be directly deduced on metallodrug targets, and to date, only a few studies have tried to establish protein targets of anticancer metallodrugs in whole-cell systems using MS-based techniques.

A combined ICP- and ESI-MS approach was reported by Allardyce *et al.* who treated *E. coli* (used as they are simpler than mammalian cells) with cisplatin and extracted the proteins and separated them by non-reducing 1D polyacrylamide gel electrophoresis (PAGE), Fig. 3.[35] The gel lanes were then analysed by LA-ICP-MS for their metal content and the band with the highest platinum response relative to the amount of protein was selected for peptide fingerprinting by ESI-MS and ESI-MS^2. The masses and sequences of these peptides were combined to produce a peptide fingerprint of a single protein that was unambiguously identified as the Outer Membrane Protein A (OmpA), which is involved in trans-membrane penetration of small solutes and in the maintenance of the cell shape with potential relevance for cisplatin cell uptake.

More recently, the binding of cisplatin and of the Ru(II)–arene compound [(η^6-*p*-cymene)$RuCl_2$(DMSO)] (not a drug candidate itself but rather a model for pharmacologically relevant Ru(II)–arene investigational drugs) to proteins from *E. coli* was reported using a technique called multidimensional

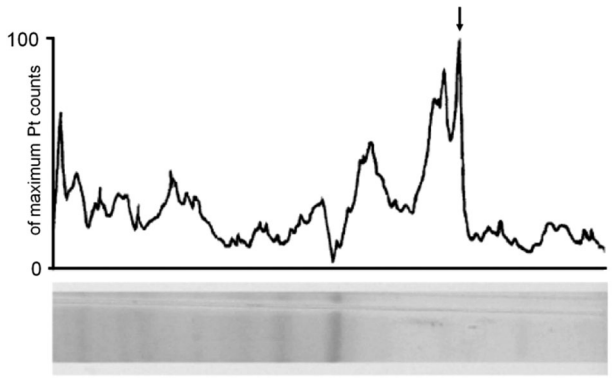


Fig. 3 An approach for the analysis of cisplatin incubated *E. coli* cells using non-reducing PAGE combined with LA-ICP-MS. The ICP-MS trace (top) shows the platinum content of the ablated gel lane (bottom). Peptide mass fingerprinting of the most intense platinum peak revealed cisplatin-binding to the outer membrane protein A. Reproduced from ref. 35 © 2001 Royal Society of Chemistry.

protein identification technology (MudPIT).[36,37] MudPIT combines strong cation exchange (SCX) and reversed-phase (RP) chromatography in a 2D separation system with ESI-MS^2 and allows up to 1500 proteins to be characterised in a 24 hours period.[38] This technique enabled the identification of 31 protein targets for cisplatin as well as determination of possible platinum binding sites.[36] Cisplatin was found on the DNA mismatch repair protein mutS, the DNA helicase II, topoisomerase I, thioredoxin 1 and two efflux proteins, and the binding sites included nucleophilic O-donors (L-aspartic and L-glutamic acid, L-serine, L-threonine, L-tyrosine) in 18 proteins and S-donors (methionine) in 9 proteins. The same approach allowed the identification of five protein targets for [(η^6-*p*-cymene)-$RuCl_2$(DMSO)]; in particular a cold-shock protein, which regulates the expression of stress-response proteins. Binding was also characterised on three stress-response proteins. In this case L-aspartic acid, L-lysine and L-threonine were identified as the probable Ru binding sites on the proteins.[37]

Similarly, MudPIT and metallomic studies were combined for an orthogonal analysis of the cellular fate of RAPTA-T (a toluene analogue of RAPTA-C) in cisplatin-sensitive and -resistant ovarian cancer cell lines.[24] First, subcellular fractionation and subsequent ICP-MS analysis showed an increased Ru(II) concentration in the particulate (containing the organelles), compared to the cytosolic, nucleic or cytoskeleton fractions. SEC-ICP-MS analysis of the particulate fraction displayed metal binding to high molecular weight species in cisplatin-sensitive cells, whereas ruthenium was predominantly found in low molecular weight species in cisplatin-resistant cells, indicating different metabolisation of the metallodrug in the two cell lines. MudPIT and label-free protein quantification yielded 74 proteins that were either up- or down-regulated. Out of these, down-regulation of histones and cytoskeletal proteins (vimentin) seems to be particularly important for the mode-of-action of RAPTA-T.

Although the MudPIT approach allows the molecular study of complex systems such as whole cells, its application has several limitations with respect to the analysis of metallodrugs: (1) the metal must exhibit a distinctive isotopic distribution that allows its reliable identification in appropriate peptide fragment ions; (2) the original protein binding sites must exhibit a high kinetic inertness towards both intra- and intermolecular transfer of the metal fragment to alternative coordination sites during the tryptic digestion; (3) the metal fragments must retain their peptide coordination sites to a significant extent, in particular under the prevailing acidic conditions (pH 2.3) required for the SCX separation; (4) the metal fragment must remain intact and coordinated to a significant number of the predicted metallated b/y fragments under MS^2 conditions; and (5) the sensitivity of the mass spectrometer must allow peptides down to about 10 fmol to be detected, corresponding to $\sim$100 copies per cell.[36]

3.2 Analysis of serum and serum proteins

Metal-based chemotherapeutics are administered intravenously and, therefore, protein constituents of the blood including human serum albumin (HSA), transferrin (Tf) and globulins are thought to play a crucial role in the delivery and storage of anticancer metallodrugs.[39] The analysis of metallodrugs in blood serum requires the use of chromatographic (such as SEC) and electrophoretic separation techniques [*e.g.* capillary zone electrophoresis (CZE)] in combination with ESI- or ICP-MS.[29] SEC has been a widely used online separation technique for the analysis of metallodrugs in serum or towards serum proteins due to certain advantages compared to other separation techniques. IEC usually requires buffers with high ionic strength and extreme pH values whereas SEC can be performed under simulated physiological conditions. The major advantage of SEC over CE lies in the ease and robustness of the hyphenation to ICP-MS, as well as better detection limits.[40] However, CE coupled to ICP-MS or other MS techniques has the important advantage of compatibility with physiological conditions, low sample consumption and high separation power. Physiologically relevant solution conditions are crucial for studying the reactivity of metallodrugs toward biomolecules to avoid interferences which might alter the metal–ligand bonds or the metal–protein adducts. Factors that may influence the reactivity include the pH of the solution, the type of buffer, the ionic strength, *etc.* Despite the investigational efforts for characterising the reactivity of metallodrugs toward serum proteins, metal binding sites are not yet unequivocally established. This is partly due to the restricted application of top-down approaches due to small mass differences between the metallated serum proteins compared to the native proteins at high charge states and is additionally complicated by the presence of disulfide bridges in the proteins. Also, serum is a highly complex biological fluid containing approximately 2000 different proteins with concentrations that span several orders of magnitude, thus complicating comprehensive analysis.

Ruthenium-based complexes and their binding to serum proteins is possibly key to their overall mode of action and has therefore been extensively studied, *e.g.* by CZE-ICP-MS.[40] The majority of the metallodrug was found attached to HSA, which was in good agreement with pharmacokinetic data obtained for KP1019 in preliminary clinical studies.[41] Plasma of KP1339-treated

mice (KP1339 is the sodium analogue of KP1019) was complementarily analysed by SEC- and CZE-ICP-MS.[42] Interestingly, both methods revealed an increase of high molecular weight proteins >100 kDa in the serum protein profile correlating with the applied dose. It was suggested that this change is probably caused by the cross-linking effect of the Ru complex. The metallodrug seems to bind preferentially to serum albumin, while the Tf-bound proportion was below the LOQ. It is noteworthy that HSA and Tf cannot be separated by SEC alone due to their similar molar masses. 2D separation is required for this purpose involving additionally ion exchange chromatography (IEC).[43]

The binding of NAMI-A to serum proteins was investigated in a slightly different manner using offline native and denaturing 1D-PAGE as well as 2D-GE separation prior to LA-ICP-MS of gel bands.[45] The results for NAMI-A were compared to cisplatin and it was deduced that the significantly lower toxicity of NAMI-A could be a consequence of differences in the mode of binding to plasma proteins, involving weaker interactions compared to cisplatin. Besides whole serum analysis, SEC-ICP-MS was also used to investigate the interactions of cisplatin and a RAPTA-C analogue with single serum proteins.[46] Remarkably, in contrast to the aforementioned study on the Ru(III) metallodrug,[42] the technique allowed complete recovery of the metals from the SEC column and to discriminate between metallodrugs and their protein adducts at femtomolar detection levels.

Different platinum compounds have been screened for their reactivity with isolated HSA using various ESI-MS approaches, and for the positively-charged multinuclear Pt complex BBR3464 non-covalent interactions were proposed (Fig. 4).[44] ESI-QToF-MS and MS^2 were also used to characterise the interaction of cisplatin with human serum apo-transferrin involving a bottom-up approach, indicating threonine-457 as a relevant binding site, which is located in the iron-binding site of the C-terminal lobe.[47,48] Similar strategies have been applied to investigate Ru(III) and Ru(II)–arene metallodrugs binding to Tf, but binding sites were not determined to date indicating that these complexes interact with the protein *via* non-covalent interactions – thus identifying at a molecular level differences between platinum and ruthenium anticancer agents.[49,50]

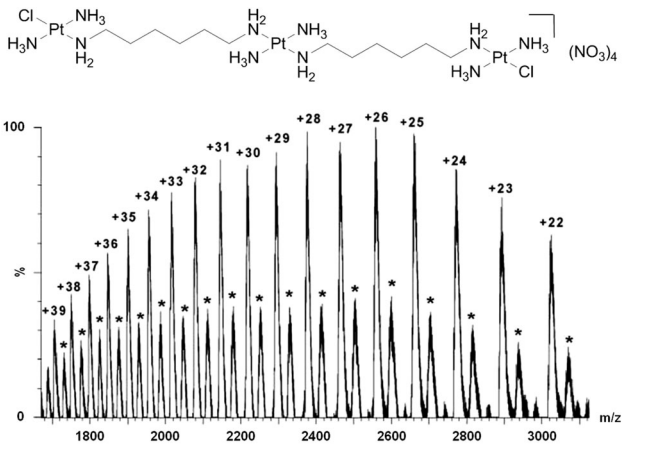


Fig. 4 Chemical formula of the triplatinum complex BBR3464 and its nESI mass spectrum after incubation with recombinant human serum albumin (rHSA). The mass difference between the metallodrug–rHSA adduct (*) and free rHSA indicates pre-association probably involving non-covalent interactions. Taken from ref. 44 © 2007 Royal Society of Chemistry.

3.3 Analysis of molecular targets

Soft ionisation techniques are required to investigate the molecular interactions of metal-based anticancer agents with biomolecules thereby ensuring that the biomolecule and the metal–biomolecule interactions remain intact. Although considered a soft ionisation technique, MALDI can induce cleavage of metal–biomolecule adducts and of other ligands attached to the metal centre.[51,52] Furthermore, its sensitivity is not very high but it can be used to determine the number of metal centres attached to biomolecules, however, no structural information can be deduced. Therefore, the nature of such metal–protein adducts formed in solution is usually characterised by ESI-MS, and ESI-MS^2 analysis is used to obtain conformational and binding site information with relevance to target identification at a molecular level.[19]

3.3.1 ESI-MS studies on protein and peptide metallodrug adduct formation. Gibson and co-workers used ESI-MS to study the reactivity of cisplatin and related compounds with ubiquitin (Ub), taken as a model system protein and, in some cases, were able to determine the competitive binding of metal complexes with other biological nucleophiles.[53–56] The high content of structural and functional information that was derived from their early ESI-MS studies prompted others to use a similar approach for the characterisation of the reactivity of metallodrugs with a wider range of proteins. Besides studies on Ub[57] and the aforementioned serum proteins responsible for metallodrug transport, investigations were carried out with potential molecular targets, *e.g.*, with metallothioneins,[58,59] which are small, cysteine-rich intracellular proteins primarily involved in storage and detoxification of soft metal ions; glutathione-*S*-transferase,[60] an important enzyme involved in the maintenance of the intracellular redox state; cytochrome *c*,[61–66] essential for electron transport in mitochondria and involved in apoptosis; or calmodulin,[67] a calcium-binding protein. Additional studies of metallodrug reactivity involved haemoglobin,[68] myoglobin,[55] superoxide dismutase,[69] lysozyme,[70] and insulin[71] in most cases serving as model proteins. The interactions of anticancer metallodrugs with proteins and peptides are often studied by LC-MS.[72,73] In general, these MS investigations revealed the nature of protein-bound metal species, adduct formation and reversibility, pseudo-kinetics of adduct formation, conformational changes upon metallation and determination of binding sites, which will be discussed in more detail for Ub.

Ubiquitin – a case study. Since most of the proteins relevant to the mode of action of metallodrugs are not readily available in purified form, the selection of a model protein is crucial for MS studies on metallodrug interaction. Moreover, the protein sequence/structure are known to reveal the accessibility of nucleophilic binding partners or binding clefts which strongly

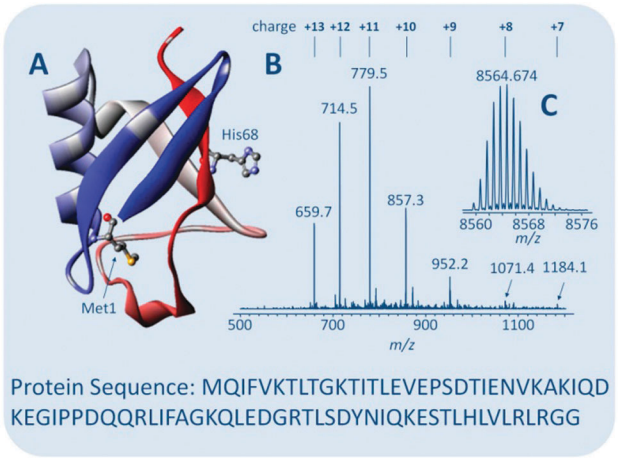


Fig. 5 (A) The tertiary structure of ubiquitin, adapted from the protein databank (1UBQ). Blue represents the N-terminus and red the C-terminus of the protein. The potential metal binding sites Met1 and His68 are highlighted. (B) The ESI-mass spectrum of ubiquitin is shown under denaturing conditions, featuring the characteristic charge envelope. (C) The deconvoluted peak shows the isotopic distribution and the accurate mass of the protein measured on an ultra-high resolution ESI-qToF-MS.

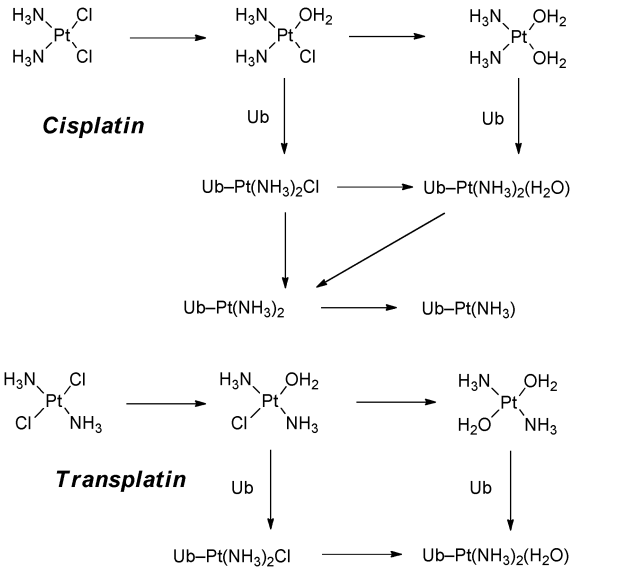


Fig. 6 Aquation of cisplatin and its anticancer inactive isomer transplatin and their adducts formed with ubiquitin (charges were omitted for clarity).

influence the extent and rate of metallodrug binding. The presence of redox-active groups may also be important for the activation/deactivation of metallodrugs. In this respect, Ub is an ideal model protein for MS analysis. It is commercially available in high purity and has a low molecular weight of ~8.6 kDa (Fig. 5). The protein sequence consists of 76 amino acids, but contains only a small number of potential binding partners including the N-terminal L-methionine (Met1; S-donor) and L-histidine at position 68 (His68; N-donor), as well as several kinetically favoured O-donors. Additionally, Ub does not contain redox-active moieties or disulfide bridges.

ESI-MS shows that the reaction of cisplatin and its anticancer inactive isomer transplatin with Ub yields different protein adducts (Fig. 6). Cisplatin forms mainly a bifunctional (*i.e.*, the Pt centre coordinated to two protein donor atoms) Ub–Pt$(NH_3)_2$ adduct with lesser amounts of monofunctional, Ub–Pt$(NH_3)_2$(OH) and Ub–Pt$(NH_3)_2$Cl and trifunctional Ub–Pt(NH_3) adducts. In contrast, transplatin gives mainly monofunctional Ub–Pt$(NH_3)_2$Cl adducts.[53] Bottom-up approaches were unsuccessful in clearly establishing the cisplatin binding site,[53] however, oxidation of Met1 prior to addition of the metal complex resulted in a decreased reactivity with cisplatin, providing indirect evidence of its preference for Met1, as did the detection of the trifunctional adduct Ub–Pt(NH_3) formed after cleavage of the ammine because of the trans effect exhibited by the Met sulphur atom.[53,54] The oxidation of Met1 had no influence on the reactivity of transplatin to Ub and His68 was proposed as the probable, preferential binding site.[55] Increasing the metal : protein molar ratio resulted in the observation of higher order adducts, *i.e.* Ub can bind three fragments derived from cisplatin. The tris-adducts possibly lead to crosslinking of Ub as revealed by their different charge envelopes under non-denaturing spraying conditions in the ESI mass spectrum.[53] The Ub–Pt$(NH_3)_2$ mono-adduct reacts with glutathione (GSH), a thiol-containing tripeptide and cellular reducing agent, to form ternary adducts, *i.e.*, a Pt-tripeptide-nucleotide species. The thiolate of GSH exhibits a strong trans-effect, leading ultimately to the cleavage of the Pt moiety from Ub.[54] In contrast, 5′-guanosine monophosphate also formed ternary adducts with Ub–Pt$(NH_3)_2$ adducts, but did not lead to Pt cleavage from Ub. Binding reversibility is a crucial factor in the mode-of-action of metallodrugs since Pt and Ru anticancer agents extensively bind to serum proteins upon intravenous administration but must be able to reach their eventual targets. Also, the ability of healthy cells to detoxify metal drugs that bind relatively weakly to their targets could be an important aspect of their selectivity mechanism. The reactivity of cisplatin, transplatin and oxaliplatin toward Ub was also studied and compared using nESI and MALDI.[51] Both ionisation methods yielded similar results, which are also in good agreement with those previously reported,[53] although MALDI resulted in a higher degree of adduct fragmentation. Oxaliplatin formed mainly Ub–Pt(chxn) adducts (chxn = 1*R*,2*R*-cyclohexanediammine), which is in accordance with activation by oxalato cleavage.

Ion mobility (IM) MS allows ions to be separated based on their mobility in a buffer gas and is strongly influenced by the protein tertiary structure. IM was therefore used to study changes in the tertiary structure of Ub upon metallation with cisplatin[74] and revealed the presence of up to three different conformations of the Ub–Pt$(NH_3)_2$ mono-adduct. The tertiary structure of proteins is highly important to exert their biological functions, such as protein–protein interactions. An additional technique for studying protein tertiary structures, which has a significant influence on its biological function, is hydrogen–deuterium exchange (HDX), but has to date only been used to study cytochrome *c* binding with anticancer metallodrugs.[66]

ESI-FT-ICR-MS^2 was applied in a top-down approach to identify the binding sites of cisplatin, transplatin and oxaliplatin

on Ub.[75] Fragmentation of the bifunctional platinum-Ub monoadducts was performed by CID and IRMPD. For both cisplatin and oxaliplatin binding to Met1 was verified with a resolution of four and two amino acids, respectively. An excerpt of the CID-MS^2 spectrum of oxaliplatin is shown in Fig. 7. It corresponds to the metallated N-terminal peptide fragment $[Pt(chxn) + MQ]^+$ verifying Met1 as the primary binding site. Transplatin was found attached to the sequence ^{19}Pro-Ser-Asp-Thr-Ile-Glu^{24} suggesting binding to oxygen donor amino acids. Interestingly, no indication for binding to His68 was obtained.[75] In a further study, the binding sites of oxaliplatin on Ub were characterised using FT-ICR- and FT-orbitrap-MS^2 in a top-down approach.[76] In particular, the fragmentation efficiencies of the MS^2 techniques CID, HCD and ETD were compared. While CID and HCD allowed the determination of Met1 as the primary binding site, only complementary ETD permitted the direct identification of Met1 and His68 as metal binding sites of the Pt(chxn) moiety on Ub. ETD also showed the best fragmentation efficiency. Notably, both binding sites were determined from a single precursor ion in the same top-down experiment.

In pseudo-kinetic studies of adduct formation, cisplatin was shown to bind faster to Ub (and also myoglobin – another relatively small protein) than transplatin.[55] Oxaliplatin reacts at a much lower rate with Ub than the chlorido complexes due to slower activation by hydrolysis. Based on the binding studies with Ub, carboplatin was initially considered to be unreactive toward proteins. However, substituting Ub with the redox active cytochrome *c* showed the formation of various Pt–protein adducts and a similar reactivity as found for cisplatin.[62]

ESI-MS was used to study the reactions of cisplatin, transplatin or RAPTA-C with a mixture of three different model proteins, namely Ub, cytochrome *c* and superoxide dismutase.[77] In particular, RAPTA-C exhibited faster binding kinetics and preferential adduct formation with Ub and cytochrome *c*. The selectivity of metallodrugs towards particular proteins is an important factor with respect to drug activity and this type of model approach could help screen putative drug candidates prior to more elaborate studies with protein extracts from cells or the direct analysis of drug–protein interactions from treated cells (note that thus far this approach has only been performed on bacteria due to the complexity of human cells).

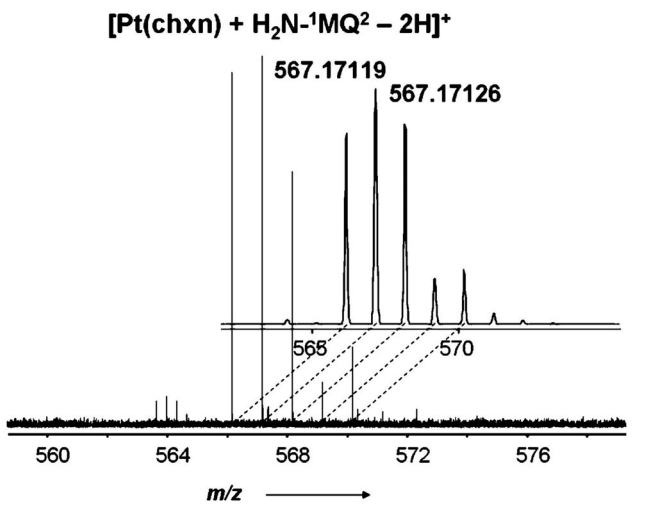


Fig. 7 Top-down binding site characterisation of oxaliplatin on ubiquitin by FT-ICR-MS^2 using CID, with the characteristic Pt isotopic distribution. Adapted with permission from ref. 75. © 2008 American Chemical Society.

It must be kept in mind that the results obtained with model systems have to be treated carefully and are highly dependent on the experimental conditions, *i.e.*, metal : protein molar ratio, type of buffer, pH, denaturing or non-denaturing spraying conditions, *etc.* The pH of the incubation solution is crucial, since most metallodrugs are activated by hydrolysis of the M–Cl bond(s) and formation of the reactive aqua complex. If the pK_a of this aqua complex is lower than the solution pH, unreactive hydroxido-species may form inhibiting coordinative binding to proteins. Due to these various factors, a correlation of metallodrug binding with pharmacological activities is difficult to establish, and it has even been demonstrated for one family of Ru(II)–arene metallodrugs that the most active in forming metallodrug–protein adducts (established by MS) is the least cytotoxic *in vitro*.[78] Nevertheless, small differences in the reactivity with proteins may give insight into the activity profile and ultimately into biological properties of metal-based anticancer agents.

3.3.2 ESI-MS studies on DNA and oligonucleotide metallodrug adduct formation. As the antiproliferative activity of platinum-based anticancer agents depends on molecular interactions with DNA by binding mainly to adjacent GG or AG moieties, virtually all available techniques including IR and NMR spectroscopy, circular dichroism, chromatography, electrophoresis, X-ray diffraction analysis, isotopic labelling and mass spectrometry as well as hyphenated techniques have been applied to study DNA–Pt interactions.[79,80] With respect to mass spectrometry the same soft ionising ESI- and MALDI-MS techniques used to study metal–protein interactions have also been used to characterise the nature of binding, (pseudo-)kinetics of binding, as well as to determine the binding sites on DNA and its models.

Initial studies investigated the interactions of Pt(II) anticancer agents with single purine bases or nucleotides as model compounds for DNA, either through direct ESI-MS[81] or online implementation of separation techniques prior to ESI- or ICP-MS.[39,82] A similar approach was followed to investigate the reactivity of RAPTA-type organometallics towards DNA model compounds,[83] whereas adduct formation of cisplatin, KP1019 and RAPTA-C with 5′-deoxyguanosine monophosphate (5′-dGMP) was analysed by CE-ESI-MS.[84,85] CE-MS studies in combination with CE-MS^2 indicated the formation of an O6,N7-bidentate binding of 5′-dGMP to cisplatin.[85] The advantage of utilising CE lies in the possibility of carrying out kinetic studies which allow the calculation of rate constants under simulated physiological conditions.

In some cases, the influence of the pH of the incubation solution on the adduct formation was also investigated, since some cancer cells may be more acidic compared to healthy cells (6.0 *vs.* 7.4) due to hypoxic conditions in solid tumours.

Notably, RAPTA-C displayed an order of magnitude increase in reactivity toward oligonucleotides at lower pH, which might contribute towards the observed selectivity of the compound toward malign cells over healthy cells. In order to determine the influence of the DNA secondary structure on the binding capabilities of metallodrugs, studies employing up to 16-mer oligonucleotides have been undertaken (albeit small fragments compared to actual DNA). Bottom-up studies using ESI and MALDI-MS on 8-mer single-stranded DNA, in which the oligonucleotide is enzymatically degraded base-by-base thereby revealing the metallodrug binding site, were combined to study the binding characteristics of the cisplatin model $[Pt(NH_3)_3(H_2O)]^{2+}$, confirming guanosine as the preferred binding site.[52] In parallel to the investigation of metallodrug–protein adducts, MALDI seems to lead at least partially to fragmentation of the adduct resulting in reduced sensitivity. The reactivity of RAPTA-C and its osmium analogue was also investigated towards a 14-mer single-stranded oligonucleotide.[86] While RAPTA-C mainly forms mono adducts accompanied by arene cleavage after 72 h, the Os analogue forms mono- and bis-adducts with retention of the arene. The reactivity of both complexes was increased toward the oligonucleotide when lowering the pH of the solution. A Ru^{II}(arene) species containing ethylenediamine was reacted with a single-stranded 14-mer oligonucleotide and formed mono- and bis-adducts corresponding to oligonucleotide–$[(\eta^6$-biphenyl)Ru(en)$]_n$ (n = 1, 2).[87] Addition of cytochrome *c* or an excess of L-histidine did not affect the reaction of the metallodrug with the oligonucleotide, underlining the target preference of this compound for DNA.

Bottom-up approaches are hampered by the use of exonucleases which usually cannot cleave modified oligonucleotides. This limitation results in incomplete data unless multiple exonucleases are used, which in turn complicates the experimental setup. Therefore, top-down MS methods for the analysis of intact oligonucleotide–metallodrug adducts using different fragmentation techniques have been developed. These studies can be carried out under almost native conditions and although positive charges usually introduced by the metallodrugs onto the negatively-charged DNA may hamper the characterisation of short DNA strands ($\leq$3 nucleotides), complete sequence coverage can usually be obtained due to complementary fragments from both sides of the strand and additional internal fragments. For example, cross-linking of adjacent GG and GTG residues by cisplatin in double-stranded 13-mer oligonucleotides was investigated by top-down ESI-FT-ICR-MS^2 using CID and IRMPD.[20] These sequence types are known to be preferred bifunctional binding sites for Pt-containing drugs on DNA. CID and IRMPD yielded similar fragmentation patterns and an excerpt of the IRMPD fragmentation is shown in Fig. 8 featuring the metallated T4:C8 fragment. It was found that cisplatin initially binds in a monofunctional fashion forming G–$Pt(NH_3)_2Cl$ adducts. Intrastrand G(T)G–$Pt(NH_3)_2$ cross-links are then formed in a further step.

The binding of different platinum and ruthenium anticancer drugs to double-stranded 13-mer and 16-mer oligonucleotides was further characterised by ESI-qToF and MALDI-MS.[21] Additional CID-ESI-MS^2 experiments revealed guanosine as a preferential

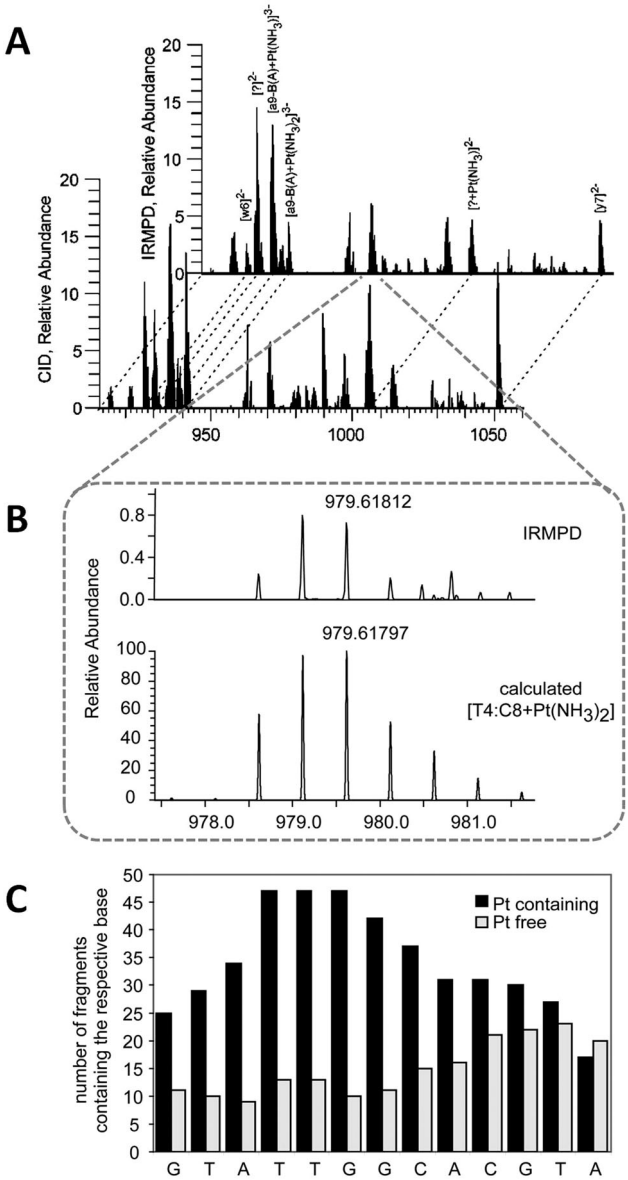


Fig. 8 (A) Top-down binding site characterisation of cisplatin on a double-stranded 13-*mer* oligonucleotide by FT-ICR-MS^2 using CID and IRMPD. (B) The zoom into the spectrum shows the characteristic Pt isotopic distribution of the fragment ion detected at *m*/*z* 979. (C) The histogram shows the single-stranded oligonucleotide sequence and the number of all assignable metallated and non-metallated fragments. Adapted with permission from ref. 20. © 2008 American Chemical Society.

binding site of Pt(II), Ru(III) and also Ru(II) anticancer metallodrugs. Interestingly, the extent of adduct formation decreased as follows: cisplatin > oxaliplatin > NAMI-A > RAPTA-T > carboplatin > KP1019. The preferred binding site and the observed trends are in excellent agreement with studies using other techniques requiring, however, higher sample amounts (NMR spectroscopy) or single crystals (X-ray diffraction analysis).

4 Conclusions and perspectives

MS serves as a powerful tool for the analysis of anticancer metallodrugs in biological samples with the advantage of short

analysis times, relatively low requirements of sample preparation and minor sample consumption as compared to other methods. Furthermore, the metals of interest usually do not occur naturally in biological samples allowing them to be detected in living systems. They also exhibit characteristic masses and isotopic distributions making them especially suited for MS analysis as this facilitates locating their specific binding sites. An array of ion sources and mass analysers in combination with separation techniques allow MS investigations of metal-based anticancer agents ranging from inter-tissue distribution to the characterisation of metallodrug–target interactions at a molecular level. While ICP-MS-based methods are used for studying metallodrug pharmacokinetics, cellular accumulation and distribution, they give no structural information, *i.e.*, it is essentially a form of elemental analysis, and hence MALDI-MS or ESI-MS^n are employed to investigate in more detail the reactions with molecular targets providing information on the exact metal–ligand adducts bound to biomolecules and even binding site details.

Correlations between molecular reactivity of metallodrugs and biological activity are hard to establish, but small differences in the reactivity of metallodrugs to biomolecules and different adduct types formed may indicate differences in their modes of action, both of which can be nicely followed by mass spectrometric methods. However, the ultimate targets and modes-of-action of anticancer metallodrugs are still elusive but modern high-resolution MS methodologies are expected to contribute significantly to the understanding of their modes of action and to the future design of metallodrugs. Its complementarity to established solution and solid state techniques makes it a valuable tool in anticancer metallodrug research.

Acknowledgements

The authors are indebted to COST actions D39, CM0902 and CM1105. S.M.M. and C.G.H. thank the ''Johanna Mahlke geb. Obermann Foundation'' for financial support. P.J.D. and C.G.H. are grateful for funding within the DACH program of the Austrian Science Fund (FWF; I496-B11) and the Swiss National Science Foundation. A.C. thanks the University of Groningen (Rosalind Franklin Fellowship) for financial support.

References

1 S. J. Lippard and J. M. Berg, *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, 1994.
2 P. J. Dyson and G. Sava, *Dalton Trans.*, 2006, 1929–1933.
3 M. A. Jakupec, M. Galanski, V. B. Arion, C. G. Hartinger and B. K. Keppler, *Dalton Trans.*, 2008, 183–194.
4 Y. W. Jung and S. J. Lippard, *Chem. Rev.*, 2007, **107**, 1387–1407.
5 C. G. Hartinger, A. A. Nazarov, S. M. Ashraf, P. J. Dyson and B. K. Keppler, *Curr. Med. Chem.*, 2008, **15**, 2574–2591.
6 C. G. Hartinger and P. J. Dyson, *Chem. Soc. Rev.*, 2009, **38**, 391–401.
7 A. Casini and L. Messori, *Curr. Top. Med. Chem.*, 2011, **11**, 2647–2660.
8 G. Gasser, I. Ott and N. Metzler-Nolte, *J. Med. Chem.*, 2011, **54**, 3–25.
9 C. G. Hartinger, N. Metzler-Nolte and P. J. Dyson, *Organometallics*, 2012, **31**, 5677–5685.
10 B. Wu, M. S. Ong, M. Groessl, Z. Adhireksan, C. G. Hartinger, P. J. Dyson and C. A. Davey, *Chem.–Eur. J.*, 2011, **17**, 3562–3566.
11 E. Meggers, *Chem. Commun.*, 2009, 1001–1010.
12 *Mass Spectrometry Handbook*, ed. M. S. Lee, John Wiley & Sons, Inc., Hoboken, 2012.
13 *Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentation, Practicalities, and Biological Applications: Fundamentals, Instrumentation, and Applications*, ed. R. B. Cole, John Wiley & Sons, Hoboken, 2nd edn, 2010.
14 K. Wrobel, K. Wrobel and J. A. Caruso, in *Applications of Physical Methods to Inorganic and Bioinorganic Chemistry*, ed. R. A. Scott and C. M. Lukehart, John Wiley & Sons, Chichester, pp. 205–225.
15 R. Lobinski, D. Schaumloffel and J. Szpunar, *Mass Spectrom. Rev.*, 2006, **25**, 255–289.
16 X. Sun, C.-N. Tsang and H. Sun, *Metallomics*, 2009, **1**, 25–31.
17 A. R. Timerbaev, C. G. Hartinger, S. S. Aleksenko and B. K. Keppler, *Chem. Rev.*, 2006, **106**, 2224–2248.
18 M. L. Nielsen, M. M. Savitski and R. A. Zubarev, *Mol. Cell. Proteomics*, 2005, **4**, 835–845.
19 R. Miao, G. Yang, Y. Miao, Y. Mei, J. Hong, C. Zhao and L. Zhu, *Rapid Commun. Mass Spectrom.*, 2005, **19**, 1031–1040.
20 A. E. Egger, C. G. Hartinger, H. Ben Hamidane, Y. O. Tsybin, B. K. Keppler and P. J. Dyson, *Inorg. Chem.*, 2008, **47**, 10626–10633.
21 M. Groessl, Y. O. Tsybin, C. G. Hartinger, B. K. Keppler and P. J. Dyson, *JBIC, J. Biol. Inorg. Chem.*, 2010, **15**, 677–688.
22 C. Silvestri and J. S. Brodbelt, *Mass Spectrom. Rev.*, 2012, DOI: 10.1002/mas.21363.
23 N. L. Kelleher, *Anal. Chem.*, 2004, **76**, 196a–203a.
24 D. A. Wolters, M. Stefanopoulou, P. J. Dyson and M. Groessl, *Metallomics*, 2012, **4**, 1185–1196.
25 J. W. Kim, B. Nie, H. Sahm, D. P. G. Brown, T. Tegeler, J.-S. You and M. Wang, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2010, **878**, 700–704.
26 M. P. Washburn, D. Wolters and J. R. Yates, *Nat. Biotechnol.*, 2001, **19**, 242–247.
27 M. Groessl and C. G. Hartinger, *Anal. Bioanal. Chem.*, 2013, **405**, 1791–1808.
28 J. Szpunar, *Anal. Bioanal. Chem.*, 2004, **378**, 54–56.
29 E. E. M. Brouwers, M. Tibben, H. Rosing, J. H. M. Schellens and J. H. Beijnen, *Mass Spectrom. Rev.*, 2008, **27**, 67–100.
30 E. Moreno-Gordaliza, C. Giesen, A. Lazaro, D. Esteban-Fernandez, B. Humanes, B. Canas, U. Panne, A. Tejedor, N. Jakubowski and M. M. Gomez-Gomez, *Anal. Chem.*, 2011, **83**, 7933–7940.
31 I. Konz, B. Fernandez, M. L. Fernandez, R. Pereiro and A. Sanz-Medel, *Anal. Bioanal. Chem.*, 2012, **403**, 2113–2125.

32 A. Bouslimani, N. Bec, M. Glueckmann, C. Hirtz and C. Larroque, *Rapid Commun. Mass Spectrom.*, 2010, **24**, 415–421.
33 R. C. Le Pla, K. J. Ritchie, C. J. Henderson, C. R. Wolf, C. F. Harrington and P. B. Farmer, *Chem. Res. Toxicol.*, 2007, **20**, 1177–1182.
34 P. Heffeter, K. Böck, B. Atil, M. Reza Hoda, W. Körner, C. Bartel, U. Jungwirth, B. Keppler, M. Micksche, W. Berger and G. Koellensperger, *JBIC, J. Biol. Inorg. Chem.*, 2010, **15**, 737–748.
35 C. S. Allardyce, P. J. Dyson, F. R. Abou-Shakra, H. Birtwistle and J. Coffey, *Chem. Commun.*, 2001, 2708–2709.
36 J. Will, W. S. Sheldrick and D. Wolters, *JBIC, J. Biol. Inorg. Chem.*, 2008, **13**, 421–434.
37 J. Will, A. Kyas, W. S. Sheldrick and D. Wolters, *JBIC, J. Biol. Inorg. Chem.*, 2007, **12**, 883–894.
38 D. A. Wolters, M. P. Washburn and J. R. Yates, *Anal. Chem.*, 2001, **73**, 5683–5690.
39 A. R. Timerbaev, C. G. Hartinger, S. S. Aleksenko and B. K. Keppler, *Chem. Rev.*, 2006, **106**, 2224–2248.
40 M. Groessl, C. G. Hartinger, K. Polec-Pawlak, M. Jarosz and B. K. Keppler, *Electrophoresis*, 2008, **29**, 2224–2232.
41 F. Lentz, A. Drescher, A. Lindauer, M. Henke, R. A. Hilger, C. G. Hartinger, M. E. Scheulen, C. Dittrich, B. K. Keppler, U. Jaehde and C. E. S. A. D. Re, *Anti-Cancer Drugs*, 2009, **20**, 97–103.
42 A. K. Bytzek, K. Boeck, G. Hermann, S. Hann, B. K. Keppler, C. G. Hartinger and G. Koellensperger, *Metallomics*, 2011, **3**, 1049–1055.
43 M. Sulyok, S. Hann, C. G. Hartinger, B. K. Keppler, G. Stingeder and G. Koellensperger, *J. Anal. At. Spectrom.*, 2005, **20**, 856–863.
44 E. I. Montero, B. T. Benedetti, J. B. Mangrum, M. J. Oehlsen, Y. Qu and N. P. Farrell, *Dalton Trans.*, 2007, 4938–4942.
45 I. Khalaila, A. Bergamo, F. Bussy, G. Sava and P. J. Dyson, *Int. J. Oncol.*, 2006, **29**, 261–268.
46 M. Groessl, M. Terenghi, A. Casini, L. Elviri, R. Lobinski and P. J. Dyson, *J. Anal. At. Spectrom.*, 2010, **25**, 305–313.
47 C. S. Allardyce, P. J. Dyson, J. Coffey and N. Johnson, *Rapid Commun. Mass Spectrom.*, 2002, **16**, 933–935.
48 I. Khalaila, C. S. Allardyce, C. S. Verma and P. J. Dyson, *ChemBioChem*, 2005, **6**, 1788–1795.
49 I. Berger, M. Hanif, A. A. Nazarov, C. G. Hartinger, R. O. John, M. L. Kuznetsov, M. Groessl, F. Schmitt, O. Zava, F. Biba, V. B. Arion, M. Galanski, M. A. Jakupec, L. Juillerat-Jeanneret, P. J. Dyson and B. K. Keppler, *Chem.–Eur. J.*, 2008, **14**, 9046–9057.
50 M. Groessl, E. Reisner, C. G. Hartinger, R. Eichinger, O. Semenova, A. R. Timerbaev, M. A. Jakupec, V. B. Arion and B. K. Keppler, *J. Med. Chem.*, 2007, **50**, 2185–2193.
51 C. G. Hartinger, W. H. Ang, A. Casini, L. Messori, B. K. Keppler and P. J. Dyson, *J. Anal. At. Spectrom.*, 2007, **22**, 960–967.
52 F. Gonnet, F. Kocher, J. C. Blais, G. Bolbach, J. C. Tabet and J. C. Chottard, *J. Mass Spectrom.*, 1996, **31**, 802–809.
53 D. Gibson and C. E. Costello, *Eur. J. Mass Spectrom.*, 1999, **5**, 501–510.
54 T. Peleg-Shulman and D. Gibson, *J. Am. Chem. Soc.*, 2001, **123**, 3171–3172.
55 T. Peleg-Shulman, Y. Najajreh and D. Gibson, *J. Inorg. Biochem.*, 2002, **91**, 306–311.
56 Y. Najajreh, T. P. Shulman, O. Moshel, N. Farrell and D. Gibson, *JBIC, J. Biol. Inorg. Chem.*, 2003, **8**, 167–175.
57 C. G. Hartinger, A. Casini, C. Duhot, Y. O. Tsybin, L. Messori and P. J. Dyson, *J. Inorg. Biochem.*, 2008, **102**, 2136–2141.
58 M. Knipp, A. V. Karotki, S. Chesnov, G. Natile, P. J. Sadler, V. Brabec and M. Vasak, *J. Med. Chem.*, 2007, **50**, 4075–4086.
59 A. Casini, A. Karotki, C. Gabbiani, F. Rugi, M. Vasak, L. Messori and P. J. Dyson, *Metallomics*, 2009, **1**, 434–441.
60 W. H. Ang, I. Khalaila, C. S. Allardyce, L. Juillerat-Jeanneret and P. J. Dyson, *J. Am. Chem. Soc.*, 2005, **127**, 1382–1383.
61 G. Yang, R. Miao, C. Jin, Y. Mei, H. Tang, J. Hong, Z. Guo and L. Zhu, *J. Mass Spectrom.*, 2005, **40**, 1005–1016.
62 A. Casini, C. Gabbiani, G. Mastrobuoni, L. Messori, G. Moneti and G. Pieraccini, *ChemMedChem*, 2006, **1**, 413–417.
63 A. Casini, C. Gabbiani, G. Mastrobuoni, R. Z. Pellicani, F. P. Intini, F. Arnesano, G. Natile, G. Moneti, S. Francese and L. Messori, *Biochemistry*, 2007, **46**, 12220–12230.
64 C. Gabbiani, A. Casini, G. Mastrobuoni, N. Kirshenbaum, O. Moshel, G. Pieraccini, G. Moneti, L. Messori and D. Gibson, *JBIC, J. Biol. Inorg. Chem.*, 2008, **13**, 755–764.
65 T. Zhao and F. L. King, *J. Am. Soc. Mass Spectrom.*, 2009, **20**, 1141–1147.
66 N. B. Zhang, Y. G. Du, M. Cui, J. P. Xing, Z. Q. Liu and S. Y. Liu, *Anal. Chem.*, 2012, **84**, 6206–6212.
67 H. L. Li, T. Y. Lin, S. L. Van Orden, Y. Zhao, M. P. Barrow, A. M. Pizarro, Y. L. Qi, P. J. Sadler and P. B. O'Connor, *Anal. Chem.*, 2011, **83**, 9507–9515.
68 R. Mandal, R. Kalke and X.-F. Li, *Rapid Commun. Mass Spectrom.*, 2003, **17**, 2748–2754.
69 S. K. Weidt, C. L. Mackay, P. R. R. Langridge-Smith and P. J. Sadler, *Chem. Commun.*, 2007, 1719–1721.
70 A. Casini, G. Mastrobuoni, C. Temperini, C. Gabbiani, S. Francese, G. Moneti, C. T. Supuran, A. Scozzafava and L. Messori, *Chem. Commun.*, 2007, 156–158.
71 E. Moreno-Gordaliza, B. Canas, M. A. Palacios and M. M. Gomez-Gomez, *Analyst*, 2010, **135**, 1288–1298.
72 M. Hahn, M. Kleine and W. S. Sheldrick, *JBIC, J. Biol. Inorg. Chem.*, 2001, **6**, 556–566.
73 M. Kleine, D. Wolters and W. S. Sheldrick, *J. Inorg. Biochem.*, 2003, **97**, 354–363.
74 J. P. Williams, H. I. A. Phillips, I. Campuzano and P. J. Sadler, *J. Am. Soc. Mass Spectrom.*, 2010, **21**, 1097–1106.
75 C. G. Hartinger, Y. O. Tsybin, J. Fuchser and P. J. Dyson, *Inorg. Chem.*, 2008, **47**, 17–19.
76 S. M. Meier, Y. O. Tsybin, P. J. Dyson, B. K. Keppler and C. G. Hartinger, *Anal. Bioanal. Chem.*, 2012, **402**, 2655–2662.
77 A. Casini, C. Gabbiani, E. Michelucci, G. Pieraccini, G. Moneti, P. J. Dyson and L. Messori, *JBIC, J. Biol. Inorg. Chem.*, 2009, **14**, 761–770.
78 S. M. Meier, M. Hanif, W. Kandioller, B. K. Keppler and C. G. Hartinger, *J. Inorg. Biochem.*, 2012, **108**, 91–95.

79 M. E. Bosch, A. J. R. Sanchez, F. S. Rojas and C. B. Ojeda, *J. Pharm. Biomed. Anal.*, 2008, **47**, 451–459.
80 M. Groessl and P. J. Dyson, *Curr. Top. Med. Chem.*, 2011, **11**, 2632–2646.
81 M. Franska, R. Franski, G. Schroeder, A. Springer, S. Beck and M. Linscheid, *Rapid Commun. Mass Spectrom.*, 2005, **19**, 970–974.
82 C. G. Hartinger and B. K. Keppler, *Electrophoresis*, 2007, **28**, 3436–3446.
83 A. Dorcier, C. G. Hartinger, R. Scopelliti, R. H. Fish, B. K. Keppler and P. J. Dyson, *J. Inorg. Biochem.*, 2008, **102**, 1066–1076.
84 P. Schluga, C. G. Hartinger, A. Egger, E. Reisner, M. Galanski, M. A. Jakupec and B. K. Keppler, *Dalton Trans.*, 2006, 1796–1802.
85 U. Warnke, C. Rappel, H. Meier, C. Kloft, M. Galanski, C. G. Hartinger, B. K. Keppler and U. Jaehde, *ChemBioChem*, 2004, **5**, 1543–1549.
86 A. Dorcier, P. J. Dyson, C. Gossens, U. Rothlisberger, R. Scopelliti and I. Tavernelli, *Organometallics*, 2005, **24**, 2114–2123.
87 F. Y. Wang, J. Bella, J. A. Parkinson and P. J. Sadler, *JBIC, J. Biol. Inorg. Chem.*, 2005, **10**, 147–155.