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RESEARCH ARTICLE

Evaluation of Nonferrous Metals as Potential In Vivo Tracers of Transferrin-Based Therapeutics

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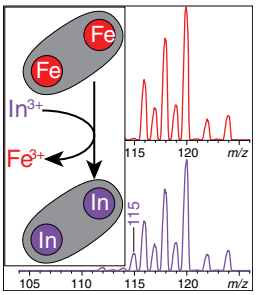

Abstract. Transferrin (Tf) is a promising candidate for targeted drug delivery. While development of such products is impossible without the ability to monitor biodistribution of Tf-drug conjugates in tissues and reliable measurements of their levels in blood and other biological fluids, the presence of very abundant endogenous Tf presents a significant impediment to such efforts. Several noncognate metals have been evaluated in this work as possible tracers of exogenous transferrin in complex biological matrices using inductively coupled plasma mass spectrometry (ICP MS) as a detection tool. Placing Ni(II) on a His-tag of recombinant Tf resulted in formation of a marginally stable protein–metal complex, which readily transfers the metal to ubiquitous physiological scavengers, such as serum albumin. An alternative strategy targeted iron-binding pockets of Tf, where cognate Fe(III) was replaced by metal ions known to bind this protein. Both Ga(III) and In(III) were evaluated, with the latter being vastly superior as a tracer (stronger binding to Tf unaffected by the presence of metal scavengers and the retained ability to associate with Tf receptor). Spiking serum with indium-loaded Tf followed by ICP MS detection demonstrated that protein quantities as low as 0.04 nM can be readily detected in animal blood. Combining laser ablation with ICP MS detection allows distribution of exogenous Tf to be mapped within animal tissue cross-sections with spatial resolution exceeding 100 μm. The method can be readily extended to a range of other therapeutics where metalloproteins are used as either carriers or payloads.

Keywords: ICP MS, Laser ablation, Tissue imaging, Targeted drug delivery, Stable isotopes

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Introduction

A very large number of potent drug candidates showing superior characteristics vis-à-vis interactions with their intended therapeutic targets in vitro nonetheless fail clinical trials because of their inability to specifically target the intended site of action. Escalating the drug dosage remains a standard remedy for this problem, but therapeutically effective doses may (and frequently do) exceed safety limits and, in extreme cases, may be fatal for the host organism. More specific delivery can be achieved in some cases by taking advantage of certain anatomical features of the targeted sites (e.g., leaky vasculature and poor drainage in many tumors, which result in preferential retention of macromolecules via enhanced permeation and retention, EPR). The possibility to exploit the EPR phenomenon generated significant enthusiasm in the field of oncology [1] and has already led to the introduction of new anti-cancer medicines (e.g., albumin-bound paclitaxel [2]). Unfortunately, the initial excitement was tempered by the limited specificity of the EPR effect (limited by the lack of cellular specificity, low vascular density, and early release of active agents [3]); tumor-to-tumor variability also limits EPR usefulness [4]. An orthogonal approach to drug delivery that is enjoying popularity in areas as diverse as oncology, neurodegenerative diseases, rare genetic disorders, and regenerative medicine takes advantage of existing transport networks within the organism. An example of a promising drug delivery system where opportunities to improve routing are frequently overlooked is transferrin (Tf), an 80 kDa iron glycoprotein, the primary function of which is the delivery of iron to cells. It is one of very few plasma proteins that gain cellular entry by the process of receptor-mediated endocytosis [5], a fact that has long attracted attention of the drug design community [6–9].

For example, malignant cells have dramatically elevated iron demands to sustain their uncontrolled growth and

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overexpress the Tf receptor (TfR), making Tf an attractive candidate for the selective delivery of cytotoxic agents to cancer cells, where cytotoxins could be either small molecule drugs chemically attached to Tf surface [8, 10, 11], macromolecules (such as Tf-diphtheria toxin conjugate TransMID [12]), or nano-objects, such as Tf-functionalized nanoparticles for siRNA delivery [13]. Above and beyond internalization via receptor-mediated endocytosis, Tf can traverse polar cells in the process of transcytosis [14], allowing it to cross physiological barriers (such as the blood-brain barrier, BBB [15] and the intestinal epithelial barrier [16]). Efforts aiming at exploitation of this unique trait for the purpose of targeted drug delivery are also underway, with the ultimate goal of developing therapeutic agents that can reach targets shielded from the vast majority of existing drugs [17, 18]. We have recently started exploring a possibility of conjugating a bacteriolytic enzyme to Tf for targeting microbial infections in the central nervous system [19, 20].

Among the many challenges faced by such efforts is the difficulty associated with the need to obtain accurate and quantitative data on biodistribution of exogenous (administered) Tf and/or Tf-based therapeutics on the background of very abundant and structurally similar (if not identical) endogenous Tf. Indeed, Tf concentration in serum of healthy human subjects ranges between 25 and 50 μM [21], at least two orders of magnitude above the serum concentration of exogenous Tf even if highly concentrated preparations are used for injection. Although radiolabeling (e.g., using ^{125}I) can be used to detect such levels of exogenous Tf [22], it requires protein modification, which may impact its receptor binding and, therefore, affect the biodistribution. Furthermore, since the endogenous Tf is expected in most relevant cases to be structurally very similar (if not identical) to the exogenous protein, this not only rules out the use of ELISA as a detection/quantitation tool (due to likely cross-reactivity), but also makes the use of many proteomics-inspired tools (e.g., using ^{18}O-labeling of proteolytic fragments [23] or entire protein [24]) highly problematic. Although in our previous work we were able to detect exogenous Tf in biological fluids using the ^{18}O-labeling strategy, we had to rely on the affinity purification step prior to MS detection taking advantage of the His-tag segment of the recombinant Tf [25]. Presence of the abundant and structurally similar endogenous Tf in tissues would also make it impossible to use traditional MALDI MS imaging approach for localization studies of exogenous Tf and Tf-based drugs in tissues, which typically relies on in situ proteolysis of proteins followed by detection of fragment peptides [26].

An alternative approach to detection and quantitation of exogenous proteins in complex biological matrices relies on placing metal tags on proteins followed by their detection with inductively coupled plasma (ICP) MS [27]. ICP MS is a sensitive technique for measuring metal- and metalloid-containing compounds [28], and in the absence of interferences from ubiquitous metals and plasma-related ions it can allow the limits of detection to be achieved at low fmol level [29, 30]. However, covalent modification of the protein surface with metal-containing tags (e.g., *p*-hydroxymercuribenzoic acid [27] or reactive metal chelates such as activated DTPA [31] and DOTA [32], which form strong complexes with multiple lanthanides [33]), is likely to exert at least some influence on protein interactions with its physiological partners. In the case of Tf and/or Tf-based drugs, covalent modification is likely to interfere with the receptor binding process, a key step in both endocytosis and transcytosis, thereby inevitably altering their trafficking patterns and rendering the biodistribution data meaningless.

Tf contains a "cognate" metal tag (iron), some isotopes of which can be detected by ICP MS with very high sensitivity, but it can hardly be used as a specific Tf tracer because of its ubiquity in all biological fluids (mostly from hemoglobin and endogenous Tf) and tissues (ferritin, myoglobin, cytochromes, etc.). While Tf is known to bind a wide range of other metals [34], the affinities vary greatly [35], and a good Tf tracer must be selected carefully. In addition to the iron binding site, we also explore the utility of the His-tag, a histidine-rich segment of the recombinant Tf (as well as many other recombinant proteins) as a potential site for metal tagging. In this study, we limited the scope of inquiry by examining two noncognate metals (indium and gallium), the interaction of which with Tf at the iron-binding sites is well-documented [35], and nickel as a metal targeting the His-tag segment of the protein [36]. Four criteria are used to select acceptable non-ferric Tf tracers with ICP MS detection: (1) the metal should form a stable complex with the protein, (2) the metal should not be removed from Tf by ubiquitous scavengers, such as serum albumin or iron-free endogenous Tf, (3) the presence of the metal should not affect the receptor binding properties of the protein, and (4) minimal mass spectral interferences from ubiquitous metals and plasma-generated ions (such as Ar^+, ArO^+, $CaOH^+$, etc.).

Materials and Methods

Preparation and Characterization of Tf Tagged with Nonferrous Metals

A diferric form of human serum transferrin (Fe_2Tf) (Intergen, Purchase, NY, USA) was converted to the apo-form (apo-Tf) by washing it with 1 mM EDTA in 3% (v/v) CH_3CO_2H, followed by buffer exchange to 50 mM HEPES buffer at pH 7.5. Indium and gallium were purchased from Alfa Aesar (Ward Hill, MA, USA). In- and Ga-tagged Tf were produced by incubating apo-Tf with metal-nitrilotriacetate (NTA) complexes in 100 mM NH_4HCO_3 and 50 mM HEPES buffer at room temperature for 1 h followed by buffer exchange to 150 mM ammonium acetate, pH 7.5. The extent of protein tagging was determined by native ESI MS using a SolariX Fourier transform ion cyclotron resonance (FT ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The possibility of metal exchange between In- and Ga-tagged Tf and apo-Tf, as well as the receptor-binding by Tf substituted with nonferrous metals, was examined by native ESI MS using a QStar XL (AB Sciex, Toronto, Canada) hybrid

quadrupole/time-of-flight mass spectrometer equipped with a standard ESI source.

Recombinant human Fe_2Tf containing an N-terminal His-tag was generously provided by Professor Anne B. Mason (University of Vermont College of Medicine), and the nickel-tagged form of this protein was produced by incubating it with significant molar excess of $NiSO_4$ in 20 mM ammonium acetate at pH 7.5. The extent of Fe_2Tf tagging with Ni^{2+} was determined by native ESI MS using a SolariX FT ICR mass spectrometer (vide supra). Unlike most other glycoproteins, Tf exhibits surprisingly little heterogeneity (see Figure S1 in Supplementary Material), which makes the analysis of metal-binding measurements carried out by native ESI MS relatively straightforward. The efficiency of nickel transfer to albumin was determined by incubating the Ni-tagged protein with bovine serum albumin (Calbiochem, La Jolla, CA, USA) prior to the ESI MS analysis.

ICP MS

Limits of detection of In-tagged Tf (In_2Tf) by ICP MS were determined by spiking 1 mL of bovine serum (Equitech-Bio, Inc., Kerrville, TX, USA) with a measured amount of In_2Tf, followed by an overnight digestion using 2 mL of freshly prepared HNO_3/H_2O_2 mixture (3:1, v:v). Rhodium (Inorganic Ventures, Christiansburg, VA, USA) was used as an internal standard and was added to the serum prior to digestion. The digested samples were diluted with deionized water to a final volume of 20 mL. A series of indium standard solutions (5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, and 0 ppb) containing 10% HNO_3/H_2O_2 and spiked with internal standard were prepared before each experiment. All ICP MS measurements were carried out using a NexION 300X ICP mass spectrometer (PerkinElmer, Waltham, MA, USA).

Animal Models

Experiments were carried out at the animal facilities of the University of Massachusetts, Amherst, on 10-wk-old male Wistar rats purchased from Charles River Laboratories (Wilmington, MA, USA). All In_2Tf samples were buffer-exchanged into 100 mM sterilized PBS buffer solution prior to injections. Rats were euthanized by CO_2 overdose 24 h after the intravenous injection of 7.5 mg/kg dose of body weight of In_2Tf. Blood samples were collected in heparin coated tube to prevent coagulation. Organs of interest were harvested, rinsed in PBS, dried, weighed, and stored in a –80°C freezer.

Imaging LA-ICP-MS

Organs of rats were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA) and cryocut at –20°C into slices of 20 µm thickness with a LEICA CM1850 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). The slices were placed on regular glass slides and air dried. Experiments were conducted with a CETAC LSX-213 G2 laser ablation system (Lincoln, NE, USA) coupled to the NexION 300X ICP mass spectrometer (vide supra). LA unit was synchronized with the ICP-MS in external triggering mode. Organ sections were inserted into the cell and ablated line by line. Elemental images were reconstructed using ImageJ, an open source image generator software (http://rsbweb.nih.gov/ij/), following the extraction and compilation of individual element list of pixels from raw data files, each of which was acquired from a single ablated line containing signal intensity values for all metals measured *(e.g.,* ^{57}Fe and ^{115}In).

Results and Discussion

Evaluation of Nickel as a Tracer of Recombinant Tf and Tf-Based Products

The recombinant form of Tf used in our work had an N-terminal His-tag, a short peptide containing a hexahistidine segment, which is frequently inserted into the wild-type protein sequence to facilitate its purification on Ni columns. Since the His-tag has high affinity to Ni^{2+} ions and at the same time does not participate in Tf interaction with its receptor, it might be considered as a possible location for placing a metal tag for ICP MS detection. Indeed, saturation of Tf solution with molar excess of $NiSO_4$ resulted in attachment of at least one metal ion to each Tf molecule, as evidenced by the disappearance of the Ni-free protein signal from the ESI mass spectrum and appearance of protein ions, masses of which are consistent with formation of Ni adducts (Figure 1). Importantly, nickel binding did not lead to a conformational change, as no high charge density ions (below *m/z* 3,000) were detected.

However, the ability of recombinant Tf to bind Ni^{2+} ions does not guarantee that this metal will be a reliable tracer of this

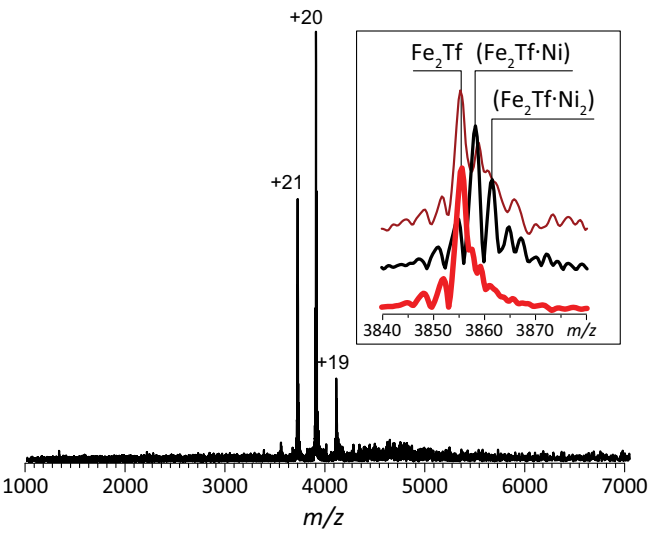

Figure 1. ESI mass spectrum of recombinant iron-saturated Tf incubated with $NiSO_4$. The inset shows ESI mass spectra of Tf before (red trace) and after (black) incubation with $NiSO_4$. The top trace shows the result of adding albumin to the solution of Tf incubated with $NiSO_4$. All mass spectra were acquired at near-physiological conditions (pH 7.5, 20 mM ammoinium acetate). Only ion peaks at charge state +20 are shown for clarity

protein in biological samples: should this metal be removed from Tf while in circulation by various scavengers, all information on Tf distribution obtained by tracing Ni using ICP MS would be meaningless. The stability of the Ni adducts of Fe_2Tf was evaluated by incubating Ni-saturated Fe_2Tf with albumin, the principal nickel binding protein in vivo [37]. Native ESI MS spectra acquired post-incubation provide unequivocal evidence that the majority of Ni^{2+} ions are readily removed from the His-Tag of recombinant Tf by albumin (Figure 1 inset, top trace), indicating that this metal cannot be used as a reliable tracer of Tf in vivo.

Evaluation of Nonferrous Metals Associating with the Protein at the Iron-Binding Sites: Gallium and Indium

Unlike nickel, which readily binds to iron-saturated Tf, a variety of metals can associate with the apo-form of Tf at the iron-binding sites [34]. Indium and gallium in particular are known to have significant Tf affinity comparable to iron [34], and endogenous Tf has been implicated in transporting radioactive isotopes of these metals used in medicine as imaging agents [38]. Although many other metals can form stable complexes with Tf, the significant difference in their ionic radii and that of Fe^{3+} results in significant distortions of the closed conformation of Tf [35, 39], which is likely to have a pronounced influence on its receptor binding properties. Since the goal of our work is to design tracers of Tf and Tf-based products that would adequately reflect their biodistribution, we limited our search to In^{3+} and Ga^{3+} as their physical characteristics are sufficiently close to Fe^{3+} and, therefore, are less likely to alter the receptor binding. Preparation of In-saturated Tf at neutral pH results in formation of a complex where association of In^{3+} with each lobe of the protein is also accompanied by binding of a synergistic ion (CO_3^{2-}), mirroring the Fe^{3+} binding to this protein. Contrary to this, binding of only a single Ga^{3+} ion to Tf (accompanied by the synergistic anion) was observed under the identical conditions when Tf solution was saturated with Ga salt. While earlier spectroscopic work provided evidence of Ga/Tf complex formation with 2:1 stoichiometry [40, 41], our results are consistent with the previous studies of Ga/Tf complexes using native ESI MS, where only 1:1 complexes were observed [35].

It may be argued that the absence of Ga_2Tf species in native ESI mass spectra could be a consequence of the failure of the metal binding to trigger cleft closure in one of the Tf lobes (which is likely to diminish the protein/metal complex stability in the gas phase, as is the case for Bi/Tf complex [35]). However, even if this is the case, and the Ga_2Tf species are indeed formed in solution but cannot be observed by native ESI MS because one of the protein lobes remains in the open conformation (characteristic of either the apo-form of Tf, or the holo-form of this protein at endosomal pH [42]), the receptor binding characteristics of such putative Ga_2Tf species would deviate from that of Fe_2Tf species, which makes Ga hardly suitable as a Tf tracer. Lastly, the Ga_xTf species are unstable vis-à-vis metal replacement in the presence of ferric ion stabilized by low-molecular weight chelators: 1-h incubation of Ga-saturated Tf in Fe·NTA solution (with 10 times molar excess of Fe·NTA) results in complete displacement of Ga^{3+} ions and formation of diferric transferrin Fe_2Tf (Figure 2a).Therefore, should Ga-labeled Tf or Tf-based therapeutic product be exposed to a pool of low-molecular weight iron post-administration, there is a high likelihood that all metal labels will be lost, making it impossible to trace the exogenous Tf using ICP MS-based methods of detection.

Unlike gallium, indium shows vastly improved characteristics vis-à-vis Tf binding. First, a dimetal form of indium-saturated Tf (In_2Tf) can be readily prepared with no traces of apo-Tf or In_1Tf. Importantly, indium-saturated Tf also exhibits markedly improved stability when exposed to a pool of low-molecular weight iron in vitro: one of the In^{3+} ions remains to be bound to the protein even after 1-h incubation of In_2Tf in solution of Fe·NTA (10 times molar excess of the latter) with no sign of formation of the diferric transferrin (Figure 2b). Lastly, In_2Tf is still capable of binding to transferrin receptor (TfR) with high affinity, as is evident from the mass spectrum of the In_2Tf/TfR mixture acquired under near-native conditions (Figure 3). The only two ionic species present in solution correspond to the free protein (which was present in slight excess) and its 2:1 complex with the receptor with no detectable signal corresponding to either free receptor or 1:1 In_2Tf/TfR complex.

While the ability of Tf to retain at least one bound In^{3+} ion even in the presence of a large pool of low-molecular weight iron is critical for the ability to track metal-labeled Tf in biological fluids and tissues, exchange of even a single In^{3+} ion from In_2Tf for ferric ion may create a pool of low-molecular weight indium (not associated with Tf), leading to false-positive signals if indium is used as a Tf reporter. Although one might expect that low-molecular weight indium should be removed from circulation very effectively by passing through the glomerular filters (in which case no false-positive signal would be generated), the circulation lifetime of indium not associated with Tf might be significantly increased as a result of binding to scavenger proteins (such as albumin), which would make quick passage through kidneys unlikely. In order to find ways to increase the stability of In_2Tf, we explored the utility of oxalate as an alternative synergistic anion. Oxalate has been shown in the past to increase iron affinity of Tf to a very significant extent [43–45], although its influence on stabilizing Tf complexes with nonferric metals remains unknown. We investigated the ability of oxalate to "lock" In^{3+} within Tf and prevent its exchange with low-molecular weight iron by preparing In_2Tf in which both carbonates acting as synergistic anions were replaced with oxalate anions (see the *Materials and Methods* section for detail). The resulting complex $(In^{3+}C_2O_4^{2-})_2Tf$ was found to be remarkably stable with respect to metal retention, showing no signs of indium-to-iron exchange after 1-h incubation in a pool of low-molecular weight iron (Figure 2c).

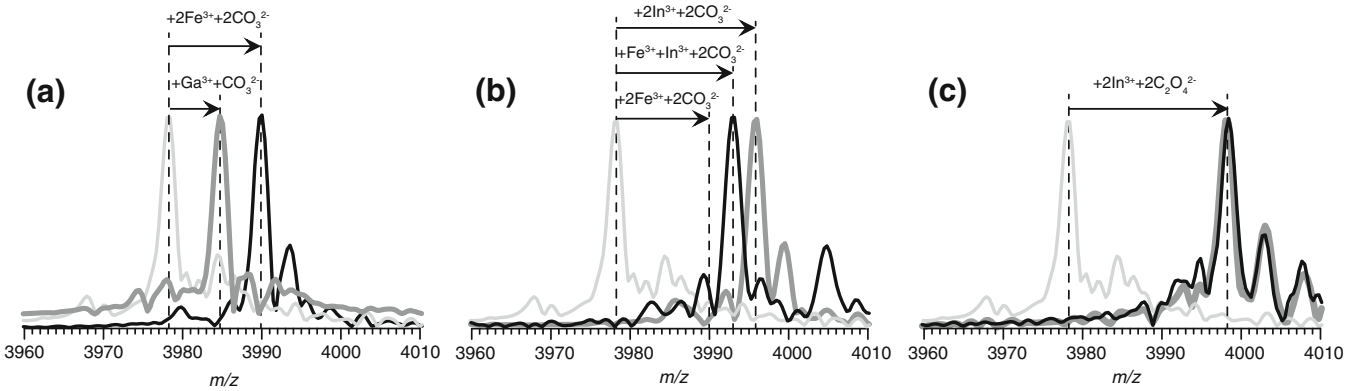

Figure 2. Replacement of noncognate metals from Tf by iron: native ESI mass spectra of Ga- (**a**) and In-saturated Tf (**b**) incubated with Fe•NTA. Only protein ion peaks at charge state +20 are shown for clarity (gray traces show reference ion peaks of apo-Tf). The effect of replacing carbonate with oxalate as a synergistic anion on metal exchange is shown in (**c**): In_2Tf ions (charge state +20) in native ESI mass spectra of In-saturated Tf before (gray) and after incubation with Fe•NTA (black)

Evaluation of Indium as a Tf Tracer in Biological Samples

Since indium has shown favorable characteristics vis-à-vis Tf binding and preserving the receptor recognition properties of this protein, it has been investigated as a tracer of exogenous Tf in biological samples both in vitro and in vivo. Possible matrix effects were evaluated and the limit of detection was determined by spiking the commercial bovine serum samples with small amounts of In_2Tf solution in 150 mM ammonium acetate, and comparing ICP MS spectra of these samples with the blank indium-free commercial serum (spiked with 150 mM ammonium acetate solution containing no In-labeled protein). Indium has two stable isotopes (^{113}In and ^{115}In), and the only two metals that can potentially interfere with indium in ICP MS are cadmium and tin. While none of these metals is expected to occur naturally in mammals, heavy metals are frequently present in solvents and reagents (e.g., traces of tin may originate from using stannous chloride $SnCl_2$, a popular pre-reducing agent, in manufacturing commercial reagents used for processing biological samples prior to ICP MS analysis). Indeed, a scan of *m/z* region 50–150 by ICP MS reveals a surprising wealth of elements that were not necessarily expected to be detected in serum (Figure 4a). Fortunately, tin was found to be the only possible interfering element contributing very low abundance isotopes to ionic signal at *m/z* 113 and 115. The origin of tin was traced to the reagent-grade hydrogen peroxide

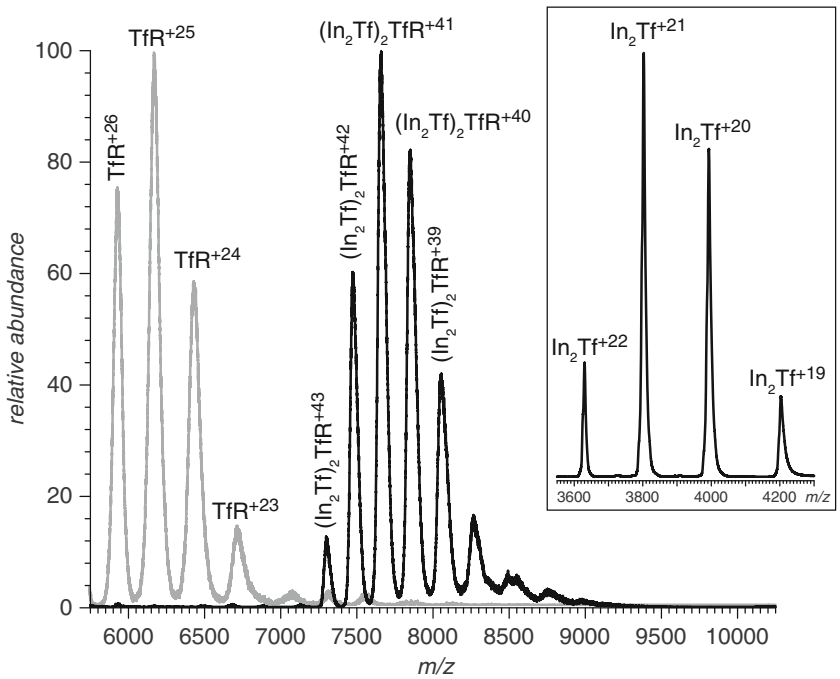

Figure 3. Nano-ESI mass spectrum of a mixture of In_2Tf (6 μM) and TfR (3 μM) in 150 mM ammonium acetate at pH 7.5. The gray trace shows the reference spectrum of TfR (acquired in the absence of transferrin). Inset show the ionic signal representing the excess of free In_2Tf in solution

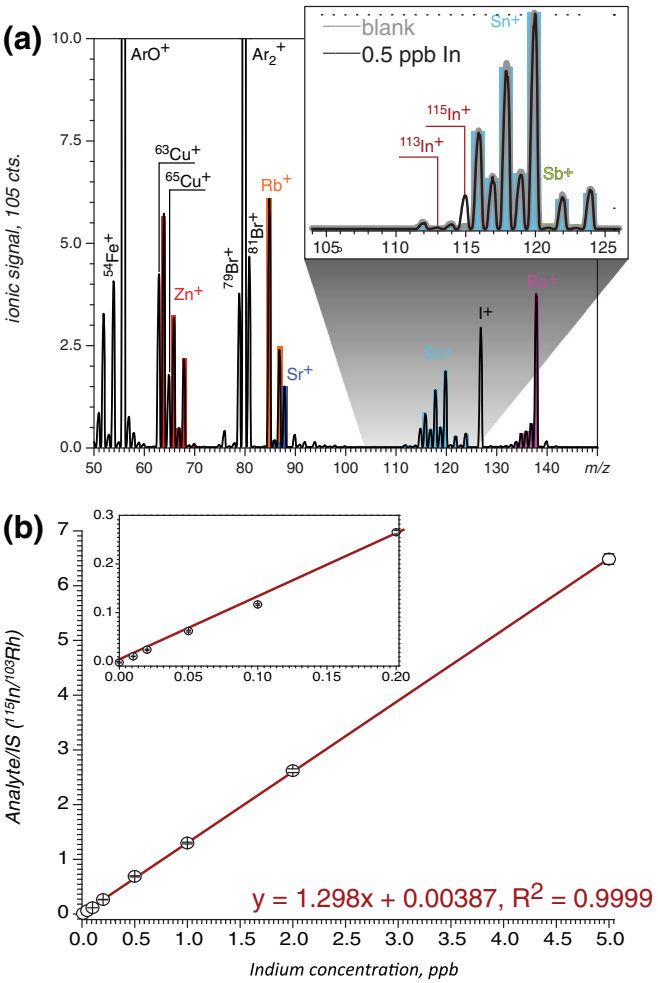

Figure 4. (**a**) ICP mass spectrum of bovine serum spiked with In_2Tf in 150 mM ammonium acetate (to a total indium concentration of 0.5 ppb). The inset shows a zoomed view of the *m/z* region 104-126 for the spiked sample (black trace) and the blank (indium-free serum sample, gray trace) normalized to the intensity of ^{120}Sn signal. Colored bars represent natural isotopic distributions of select elements (labeled with the same color). (**b**) Calibration curve for indium (^{115}In) in serum obtained with rhodium as an internal standard. The inset is a zoomed view of the calibration curve below 0.2 ppb of indium

used for sample digestion (see Figure S2 in Supplementary Material for more detail), and it might be possible to eliminate this interfering species from the ICP mass spectra altogether by selecting a higher-quality supplier of this reagent. Short of complete elimination of tin from the reagents, a correction to the intensities of ^{113}In and ^{115}In can be readily made based on the known abundance distribution of tin isotopes and the intensity of ^{116}Sn, ^{117}Sn, and other high-abundance tin isotopes in the ICP mass spectrum.

The signal for the most abundant indium isotope (^{115}In) was clearly discernible in the ICP mass spectrum of In_2Tf-spiked commercial serum sample where the final concentration of indium was as low as 0.01 ppb (corresponding to 0.04 nM protein concentration). A calibration curve for indium in serum constructed using rhodium as an internal standard (Figure 4b) shows excellent linearity ($R^2 > 0.99$) up to 5 ppb, suggesting that In-labeling can be used for sensitive detection and quantitation of exogenous Tf and Tf-based therapeutics in biological fluids and tissue homogenates.

Measuring indium concentration in blood, cerebrospinal fluid, and other biological fluids following administration of Tf-based therapeutics labeled with this metal will be invaluable for obtaining reliable pharmacokinetic profiles of these protein drugs. However, a detailed picture of biodistribution of Tf-based therapeutics cannot be reconstructed from the average concentrations of indium in tissue homogenates, where all information on uneven distribution of the protein therapeutic across organ tissues would be lost during the homogenization process. However, biodistribution patterns of metals in organ cross-sections can be obtained by combining ICP MS with laser ablation (LA) [46], a technique that emerged in the past several years as a powerful tool to probe distribution of biometals in various tissues [47]. In order to assess the feasibility of using LA-ICP MS as a means of obtaining biodistribution patterns of Tf-based therapeutics in tissues, a metal distribution map was generated for a cross-section of rat spleen harvested 24 h after the injection of In_2Tf. The medium-resolution distribution maps of iron (^{57}Fe) and indium (^{115}In) differ significantly in absolute intensities, but the distribution patterns are very similar (Figure 5), suggesting that the sensitivity provided by LA-ICP MS is adequate for mapping biodistribution of Tf-based therapeutics under clinically relevant conditions.

While detection and quantitation of proteins (including biopharmaceuticals) in biological fluids can be carried out using commonly accepted approaches relying on introducing isotopic labels (e.g., ^{18}O) either at the peptide [23] or the whole protein level [24], application of these procedures to Tf-based therapeutics is problematic because of the presence of very

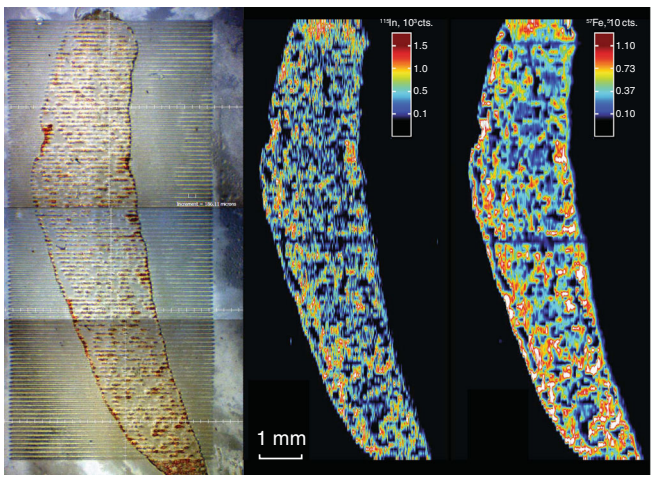

Figure 5. LA-ICP MS imaging of iron (^{57}Fe) and indium (^{115}In) distribution in the cross-section of spleen harvested from a rat 24 h after the injection of In_2Tf. Shown on the left is a photograph of the spleen cross-section following completion of the imaging work with laser-etched lines

abundant endogenous protein, the amino acid sequence of which is very similar if not identical to that of the exogenous Tf. Therefore, the ability to selectively detect and quantitate exogenous protein will hinge on the ability to detect a proteolytic fragment unique to that protein, but absent from endogenous Tf. An alternative strategy would take advantage of a non-native segment within the recombinantly produced exogenous protein (such as a His-tag) to afford selective capture of that protein [48]. However, even in these circumstances, we have found that the sensitivity afforded by ICP MS measurements of In_2Tf exceeds that of the commonly accepted techniques combining ^{18}O labeling and LC/MS detection by at least factor of three.

The advantages provided by indium labeling and ICP MS detection are even more significant when it comes to measuring biodistribution patterns of Tf-based biopharmaceuticals. While molecular imaging with MALDI MS [49] can produce protein distribution patterns in tissue cross-sections, the size of Tf places it outside of the mass range amenable to direct detection with MALDI TOF MS, and requires in situ digestion to be carried out prior to imaging [50]. The very low levels of exogenous Tf in tissues under clinically relevant conditions and the presence of abundant endogenous Tf are very likely to prevent successful utilization of traditional MALDI MS imaging for characterization of biodistribution of Tf-based therapeutics in animal tissues.

Finally, indium labeling provides a possibility to multiplex the measurements, another very important advantage for both Tf quantitation and imaging studies. Although in this work all measurements were based on the intensity of ^{115}In, both stable isotopes of this metal are available commercially, and it is possible to produce and co-inject isotope-coded variants of Tf-based therapeutics and selectively track them in a single animal.

Conclusions

The spectacular progress in the field of ICP MS in the past decade enabled probing of biometals (such as metals associated with proteins) in biological samples at unprecedented levels of detail, including sensitive detection and quantitation as well as spatially resolved measurements (2D-imaging) in tissues. Tf is a ferroprotein that is used as a delivery vehicle in a range of therapies that are currently under development, and availability of a reliable analytical method to trace exogenous Tf in various biological samples would be a boon to the drug development efforts. While the cognate metal (iron) is hardly suitable for the role of Tf tracer because of its high abundance in all living organisms, it can be replaced with nonferrous metals, some of which can be used as Tf tracers. Numerous ESI MS-based methods have been developed in the past two decades to probe protein/metal complexes at a variety of levels [45, 51–56], and many of these techniques have been used in this work to investigate three metals (Ni, Ga, and In) as potential tracers of Tf. One of them (In) has been found to be ideally suited for this role, as it binds strongly to both lobes of Tf, and allows the latter to retain its receptor-binding properties. Absence of strong interferences in ICP mass spectra allows indium to be detected with high sensitivity in complex biological matrices, enabling both quantitation of In_2Tf in biological fluids and tissue homogenates, and imaging of this protein in organ cross-sections. Although this work focuses on intact Tf (rather than Tf/drug conjugates), a follow-up paper will present the results of studies of biodistribution of a Tf-based antimicrobial agent (a Tf/bacteriolytic enzyme conjugate [19]) designed to target infections on the central nervous systems that are shielded from many antibiotics by a blood-brain barrier.

Further studies will be needed to determine if other nonferrous metals from the extensive list of Tf binders [34, 57] as well as iron isotopes [58–60] can be used as tracers of this protein. Expanding the repertoire of Tf tracers will enable multiplexing in biodistribution studies of exogenous Tf and Tf-based therapies. Above and beyond Tf, the strategy outlined in this work may also be used to facilitate pharmacokinetic and biodistribution studies of other drug candidates that utilize metalloproteins as vehicles for targeted delivery (such as the hemoglobin/haptoglobin complex [61]).

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References

1. Bertrand, N., Wu, J., Xu, X.Y., Kamaly, N., Farokhzad, O.C.: Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. Adv. Drug Deliv. Rev. **66**, 2–25 (2014)
2. Kratz, F.: A clinical update of using albumin as a drug vehicle—a commentary. J. Control Release. **190**, 331–336 (2014)
3. Nehoff, H., Parayath, N.N., Domanovitch, L., Taurin, S., Greish, K.: Nanomedicine for drug targeting: strategies beyond the enhanced permeability and retention effect. Int. J. Nanomed. **9**, 2539–2555 (2014)
4. Ruoslahti, E., Bhatia, S.N., Sailor, M.J.: Targeting of drugs and nanoparticles to tumors. J. Cell Biol. **188**, 759–768 (2010)
5. Luck, A.N., Mason, A.B.: Transferrin-Mediated Cellular Iron Delivery. In: Lutsenko S, Arguello JM, Eds. Elsevier Academic Press Inc.: San Diego, (2012)
6. Li, H., Sun, H., Qian, Z.M.: The role of the transferrin-transferrin-receptor system in drug delivery and targeting. Trends Pharmacol. Sci. **23**, 206–209 (2002)
7. Qian, Z.M., Li, H., Sun, H., Ho, K.: Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol. Rev. **54**, 561–587 (2002)
8. Daniels, T.R., Delgado, T., Helguera, G., Penichet, M.L.: The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. Clin. Immunol. **121**, 159–176 (2006)
9. Luck, A.N., Mason, A.B.: Structure and dynamics of drug carriers and their interaction with cellular receptors: Focus on serum transferrin. Adv. Drug Deliv. Rev. **65**, 1012–1019 (2013)

10. Head, J.F., Wang, F., Elliott, R.L.: Antineoplastic drugs that interfere with iron metabolism in cancer cells. Adv. Enzym. Regul. **37**, 147–169 (1997)
11. Daniels, T.R., Bernabeu, E., RodrÃguez, J.A., Patel, S., Kozman, M., Chiappetta, D.A., Holler, E., Ljubimova, J.Y., Helguera, G., Penichet, M.L.: The transferrin receptor and the targeted delivery of therapeutic agents against cancer. Biochim. Biophys. Acta **1820**, 291–317 (2012)
12. Rainov, N.G., Soling, A.: Technology evaluation: TransMID, KS Biomedix/Nycomed/Sosei/PharmaEngine. Curr. Opin. Mol. Ther. **7**, 483–492 (2005)
13. Davis, M.E.: The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. Mol. Pharm. **6**, 659–668 (2009)
14. Broadwell, R.D., Baker-Cairns, B.J., Friden, P.M., Oliver, C., Villegas, J.C.: Transcytosis of protein through the mammalian cerebral epithelium and endothelium: III. Receptor-mediated transcytosis through the blood-brain barrier of blood-borne transferrin and antibody against the transferrin receptor. Exp. Neurol. **142**, 47–65 (1996)
15. Pardridge, W.M.: Re-engineering biopharmaceuticals for delivery to brain with molecular Trojan horses. Bioconj. Chem. **19**, 1327–1338 (2008)
16. Amet, N., Wang, W., Shen, W.-C.: Human growth hormone-transferrin fusion protein for oral delivery in hypophysectomized rats. J. Control Release **141**, 177–182 (2010)
17. Jones, A.R., Shusta, E.V.: Blood-brain barrier transport of therapeutics via receptor-mediation. Pharm. Res. **24**, 1759–1771 (2007)
18. Smith, M.W., Gumbleton, M.: Endocytosis at the blood-brain barrier: from basic understanding to drug delivery strategies. J. Drug Target **14**, 191–214 (2006)
19. Nguyen, S.N., Bobst, C.E., Kaltashov, I.A.: Mass spectrometry-guided optimization and characterization of a biologically active transferrin-lysozyme model drug conjugate. Mol. Pharm. **10**, 1988–2007 (2013)
20. Kaltashov, I.A., Bobst, C.E., Nguyen, S.N., Wang, S.: Emerging mass spectrometry-based approaches to probe protein-receptor interactions: Focus on overcoming physiological barriers. Adv. Drug Deliv. Rev. **65**, 1020–1030 (2013)
21. Burtis, C.A., Ashwood, E.R., Tietz, N.W.: Tietz textbook of clinical chemistry, 3rd ed. W.B. Saunders: Philadelphia (1999)
22. Mayle, K.M., Le, A.M., Kamei, D.T.: The intracellular trafficking pathway of transferrin. Biochim. Biophys. Acta **1820**, 264–281 (2012)
23. Fenselau, C., Yao, X.: $^{18}O_2$-Labeling in quantitative proteomic strategies: a status report. J. Proteome Res. **8**, 2140–2143 (2009)
24. Wang, S., Kaltashov, I.A.: A new strategy of using O^{18}-labeled iodoacetic acid for mass spectrometry-based protein quantitation. J. Am. Soc. Mass Spectrom. **23**, 1293–1297 (2012)
25. Wang, S., Bobst, C., Kaltashov, I.: A new liquid chromatography–mass spectrometry-based method to quantitate exogenous recombinant transferrin in cerebrospinal fluid: a potential approach for pharmacokinetic studies of transferrin-based therapeutics in the central nervous systems. Eur. J. Mass Spectrom. **21**, 369–376 (2015)
26. Angel, P.M., Caprioli, R.M.: Matrix-assisted laser desorption ionization imaging mass spectrometry: in situ molecular mapping. Biochemistry **52**, 3818–3828 (2012)
27. Kutscher, D.J., del Castillo Busto, M.E., Zinn, N., Sanz-Medel, A., Bettmer, J.: Protein labeling with mercury tags: fundamental studies on ovalbumin derivatised with p-hydroxymercuribenzoic acid (pHMB). J. Analyt. Atom. Spectrom. **23**, 1359–1364 (2008)
28. Pröfrock, D., Prange, A.: Inductively coupled plasma-mass spectrometry (ICP-MS) for quantitative analysis in environmental and life sciences: a review of challenges, solutions, and trends. Appl. Spectrosc. **66**, 843–868 (2012)
29. Sanz-Medel, A., Montes-Bayon, M., Bettmer, J., Fernandez-Sanchez, M.L., Encinar, J.R.: ICP-MS for absolute quantification of proteins for heteroatom-tagged, targeted proteomics. TrAC Trends Anal. Chem. **40**, 52–63 (2012)
30. Liu, R., Lv, Y., Hou, X., Yang, L., Mester, Z.: Protein quantitation using Ru-NHS ester tagging and isotope dilution high-pressure liquid chromatography-inductively coupled plasma mass spectrometry determination. Anal. Chem. **84**, 2769–2775 (2012)
31. Patel, P., Jones, P., Handy, R., Harrington, C., Marshall, P., Evans, E.H.: Isotopic labelling of peptides and isotope ratio analysis using LC-ICP-MS: a preliminary study. Anal. Bioanal. Chem. **390**, 61–65 (2008)
32. Ahrends, R., Pieper, S., Kuhn, A., Weisshoff, H., Hamester, M., Lindemann, T., Taubner, K., Linscheid, M.W.: A metal-coded affinity tag approach to quantitative proteomics. Mol. Cell. Proteomics **6**, 1907–1916 (2007)
33. Bunzli, J.C.G.: Benefiting from the unique properties of lanthanide ions. Acc. Chem. Res. **39**, 53–61 (2006)
34. Sun, H., Li, H., Sadler, P.J.: Transferrin as a metal ion mediator. Chem. Rev. **99**, 2817–2842 (1999)
35. Zhang, M., Gumerov, D.R., Kaltashov, I.A., Mason, A.B.: Indirect detection of protein-metal binding: Interaction of serum transferrin with In^{3+} and Bi^{3+}. J. Am. Soc. Mass Spectrom. **15**, 1658–1664 (2004)
36. Hochuli, E., Dobeli, H., Schacher, A.: New metal chelate adsorbent selective for proteins and peptides containing neighboring histidine-residues. J. Chromatogr. **411**, 177–184 (1987)
37. Glennon, J.D., Sarkar, B.: Nickel(II) transport in human blood serum. Studies of nickel(II) binding to human albumin and to native-sequence peptide and ternary-complex formation with L-histidine. Biochem. J. **203**, 15–23 (1982)
38. Wadas, T.J., Wong, E.H., Weisman, G.R., Anderson, C.J.: Coordinating radiometals of copper, gallium, indium, yttrium, and zirconium for PET and SPECT imaging of disease. Chem. Rev. **110**, 2858–2902 (2010)
39. Vidaud, C., Gourion-Arsiquaud, S., Rollin-Genetet, F., Torne-Celer, C., Plantevin, S., Pible, O., Berthomieu, C., Quéméneur, E.: Structural consequences of binding of UO_2^{2+} to apotransferrin: can this protein account for entry of uranium into human cells? Biochemistry **46**, 2215–2226 (2007)
40. Harris, W.R., Pecoraro, V.L.: Thermodynamic binding constants for gallium transferrin. Biochemistry **22**, 292–299 (1983)
41. Li, Y., Liu, B., Ge, Z., Yang, B.: Spectroscopic analysis of the interaction between gallium(III) and apoovotransferrin. J. Photochem. Photobiol. B. Biol. **91**, 137–142 (2008)
42. Bobst, C.E., Zhang, M., Kaltashov, I.A.: Existence of a noncanonical state of iron-bound transferrin at endosomal pH revealed by hydrogen exchange and mass spectrometry. J. Mol. Biol. **388**, 954–967 (2009)
43. Gumerov, D.R., Kaltashov, I.A.: Dynamics of iron release from transferrin N-lobe studied by electrospray ionization mass spectrometry. Anal. Chem. **73**, 2565–2570 (2001)
44. Halbrooks, P.J., Mason, A.B., Adams, T.E., Briggs, S.K., Everse, S.J.: The oxalate effect on release of iron from human serum transferrin explained. J. Mol. Biol. **339**, 217–226 (2004)
45. Kaltashov, I.A., Bobst, C.E., Zhang, M., Leverence, R., Gumerov, D.R.: Transferrin as a model system for method development to study structure, dynamics and interactions of metalloproteins using mass spectrometry. Biochim. Biophys. Acta **1820**, 417–426 (2012)
46. Becker, J.S., Zoriy, M., Matusch, A., Wu, B., Salber, D., Palm, C.: Bioimaging of metals by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Mass Spectrom. Rev. **29**, 156–175 (2010)
47. Becker, J.S., Matusch, A., Wu, B.: Bioimaging mass spectrometry of trace elements— recent advance and applications of LA-ICP-MS: a review. Anal. Chim. Acta **835**, 1–18 (2014)
48. Wang, S., Bobst, C.E., Kaltashov, I.A.: A new LC-MS based method to quantitate exogenous recombinant transferrin in cerebrospinal fluid: a potential approach for pharmacokinetic studies of transferrin-based therapeutics in the central nervous system. Eur. J. Mass Spectrom. **21**, 369–376 (2015)
49. Lietz, C.B., Gemperline, E., Li, L.: Qualitative and quantitative mass spectrometry imaging of drugs and metabolites. Adv. Drug Deliv. Rev. **65**, 1074–1085 (2013)
50. Gessel, M.M., Norris, J.L., Caprioli, R.M.: MALDI imaging mass spectrometry: spatial molecular analysis to enable a new age of discovery. J. Proteom. **107**, 71–82 (2014)
51. Yu, X., Wojciechowski, M., Fenselau, C.: Assessment of metals in reconstituted metallothioneins by electrospray mass spectrometry. Anal. Chem. **65**, 1355–1359 (1993)
52. Zaia, J., Fabris, D., Wei, D., Karpel, R.L., Fenselau, C.: Monitoring metal ion flux in reactions of metallothionein and drug- modified metallothionein by electrospray mass spectrometry. Protein Sci. **7**, 2398–2404 (1998)
53. Fabris, D., Fenselau, C.: Characterization of allosteric insulin hexamers by electrospray ionization mass spectrometry. Anal. Chem. **71**, 384–387 (1999)
54. Kaltashov, I.A., Zhang, M., Eyles, S.J., Abzalimov, R.R.: Investigation of structure, dynamics, and function of metalloproteins with electrospray ionization mass spectrometry. Anal. Bioanal. Chem. **386**, 472–481 (2006)
55. Gumerov, D.R., Mason, A.B., Kaltashov, I.A.: Interlobe communication in human serum transferrin: metal binding and conformational dynamics investigated by electrospray ionization mass spectrometry. Biochemistry **42**, 5421–5428 (2003)

56. Hartinger, C.G., Groessl, M., Meier, S.M., Casini, A., Dyson, P.J.: Application of mass spectrometric techniques to delineate the modes-of-action of anticancer metallodrugs. Chem. Soc. Rev. **42**, 6186–6199 (2013)
57. Li, H., Sadler, P.J., Sun, H.: Rationalization of the strength of metal binding to human serum transferrin. Eur. J. Biochem. **242**, 387–393 (1996)
58. Konz, I., Fernández, B., Fernández, M.L., Pereiro, R., Sanz-Medel, A.: Absolute quantification of human serum transferrin by species-specific isotope dilution laser ablation ICP-MS. Anal. Chem. **83**, 5353–5360 (2011)
59. del Castillo Busto, M.E., Montes-Bayón, M., Sanz-Medel, A.: Accurate determination of human serum transferrin isoforms: exploring metal-specific isotope dilution analysis as a quantitative proteomic tool. Anal. Chem. **78**, 8218–8226 (2006)
60. Feng, L., Zhang, D., Wang, J., Shen, D., Li, H.: A novel quantification strategy of transferrin and albumin in human serum by species-unspecific isotope dilution laser ablation inductively coupled plasma mass spectrometry (ICP-MS). Anal. Chim. Acta. **884**, 19–25 (2015)
61. Nielsen, M.J., Moestrup, S.K.: Receptor targeting of hemoglobin mediated by the haptoglobins: roles beyond heme scavenging. Blood **114**, 764–771 (2009)