Food & Function
Linking the chemistry and physics of food with health and nutrition

Food science and nutrition is a highly multidisciplinary area. We know it can be difficult to keep abreast of each other’s work, especially when there is not enough time in the day and the pile of work keeps growing.

Wouldn’t it be great if there was a journal which pulled together high impact chemical and physical research linking to human health and nutrition? Just one platform to find what you need in the field, and reach exactly the right audience when you publish your work.

Food & Function provides a dedicated venue for physicists, chemists, biochemists, nutritionists and other health scientists focusing on work related to the interaction of food components with the human body.

Go to the website now to submit your research and register for free access!
Instrumental comparison of the determination of Cr$^{3+}$ uptake by human transferrin

C. Derrick Quarles Jr., Julia L. Brumaghim and R. Kenneth Marcus*

Received 27th July 2010, Accepted 6th October 2010
DOI: 10.1039/c0mt00032a

UV-VIS absorbance, inductively coupled plasma-optical emission spectroscopy (ICP-OES), and particle beam/hollow cathode-optical emission spectroscopy (PB/HC-OES) are presented as techniques for determining Cr$^{3+}$ loading into transferrin (Tf), with and without Fe$^{3+}$. The methods are compared based on loading percentages (i.e. 100% loading would be equal to 2 M$^{n+}$:1 Tf) determined for Cr$^{3+}$ loading into apo-transferrin. Spectral interferences and overlapping LMCT bands cause inaccurate chromium (qualitative) and iron (qualitative and quantitative) results for the UV-VIS absorbance method. The ICP-OES and PB/HC-OES methods are in good agreement providing evidence that the PB/HC-OES method is a valid technique for investigating metal–protein complexes. Maximum Cr$^{3+}$ loading into apo-transferrin over a 24 h period was determined to be 26.8 ± 3.5% by the ICP-OES method and 25.3 ± 2.2% by the PB/HC-OES method. Loading percentages were increased to 49.7 ± 1.9% (ICP-OES) and 55.7 ± 3.2% (PB/HC-OES) when the metal-transferrin solution was allowed to incubate for up to 10 days. Under non-excess carbonate conditions the Cr$^{3+}$ loading is elevated over a 24 h incubation time, but under physiological conditions the loading is inhibited. Equal loading of Fe$^{3+}$ and Cr$^{3+}$ into apo-transferrin was achieved when chromium was at a level more than 5 times in excess of iron. Inhibition of Cr$^{3+}$ loading was only observed when an excess of Fe$^{3+}$ was available to bind into apo-transferrin. The ability for Cr$^{3+}$ to displace Fe$^{3+}$ from holo-transferrin was observed as small amounts of Cr$^{3+}$ were loaded into the once occupied metal binding site.

Introduction

Metallomics is the field of research that involves understanding how metal species interact and function within biological systems. Metal containing proteins, known as metalloproteins, make up approximately one-third of all known proteins and the understanding of how these metals are trafficked in the body is the ultimate goal. The transport proteins albumin and transferrin are found in human serum and are responsible for much of the metal trafficking and uptake of essential metals required by the body. Transferrin (Tf), an iron transport protein, is found at a concentration of ~35 μM in human serum. Transferrin consists of two almost identical lobes known as the N- and C-terminal lobes that can each bind a single Fe$^{3+}$ ion. Upon iron binding to apo-transferrin (the metal free form), the metal coordinates in a distorted octahedral geometry bound to four amino acids (histidine, aspartic acid, and two tyrosines) and a synergistic anion, most commonly carbonate, acting as a bidentate ligand. It has been found that iron only binds to approximately 30% of the transferrin circulating in the serum of the human body. As such, the ~70% of the remaining transferrin (i.e., apo- or monomono-forms) is available to potentially bind other metal ions found in human serum. These metals may be introduced into the body by diet, supplementation, implants, or contaminants from the environment. Al$^{3+}$, Cr$^{3+}$, Cu$^{2+}$, Ga$^{3+}$, Ni$^{2+}$, Ti$^{4+}$, and Zn$^{2+}$ are all known to bind to transferrin, with the stability constants for these metal–protein complexes generally following the same trend as the stability constants for metal-hydroxide binding.

Understanding the role of Cr$^{3+}$ on human health has been a topic of much debate over recent years. Chromium(III) is considered an essential nutrient for carbohydrate metabolism and is thought to enhance insulin binding and activity. Transferrin is believed to be the major transporter for delivering Cr$^{3+}$ from serum to the cell. It has been determined that Cr$^{3+}$ binds to apo-transferrin with binding constants of $K_1 = 1.42 \times 10^{10}$ M$^{-1}$ and $K_2 = 2.06 \times 10^{5}$ M$^{-1}$. It has been proposed that chromium-loaded transferrin will bind to the transferrin receptors on the cell membrane and through receptor mediated endocytosis the metal ion will be released into the cell, where it is believed that chromodulin sequesters the Cr$^{3+}$ ion. Reports have shown that increases in serum insulin result in an increased amount of transferrin receptor on the cell surface, which could lead to excess uptake of transferrin-bound metal ions. Vincent posed the question as to whether increased loading of transferrin with iron prevents adequate chromium binding and transfer by transferrin, resulting in insulin resistance and diabetes. Along those same lines, it has been proposed that excessive amounts of Cr$^{3+}$ ions in the body from corrosion of metal prosthetic implants may impede the necessary iron binding and/or uptake for cell survival.

In order to study the effects of Cr$^{3+}$ binding to transferrin under various conditions, proper chemical instrumentation is...
required. Most transferrin studies involving a single-metal utilize UV-VIS absorbance techniques to determine protein concentration and the ligand-to-metal charge transfer (LMCT) bands (tyrosine to metal) to characterize the complex.5,10,20,21 Although a very simple and useful technique, UV-VIS does not provide species-specific (ion-complex) signatures, therefore it must be coupled with some form of atomic spectroscopy in order to distinguish between multiple metal ions that may be bound. Inductively coupled plasma-optical emission spectroscopy or mass spectrometry (ICP-OES or -MS) can provide the ability to detect the multiple metals bound to transferrin with very good precision and sensitivity. The primary drawback is that most ICP sources are operated at atmospheric pressure and are unable to detect H, C, N, or O responses which reflect the composition and concentration of proteins. The stoichiometry of metal to protein composition is what ultimately is needed to determine loading percentages for metal loaded protein studies. The Sanz-Medel group recently reported the interactions of cisplatin with serum proteins using S responses (reflective of protein concentration) via ICP-MS to identify protein-metal complexes.22 Sulfur and phosphorous signals are widely interfered with by polyatomic ions and are not sensitive due to high ionization potentials, making their determinations difficult with conventional ICP-MS instrumentation.23 Thus in order to determine protein concentrations and loading abilities in most studies, both ICP and UV-VIS are used jointly.

As an alternative, electrospray ionization-mass spectrometry/mass spectrometry (ESI-MS/MS) can be used as a qualitative method to determine whether or not metal is bound within the protein, but ESI-MS offers poor quantification capabilities.24 Recently ESI-MS was used to determine reactivity and selectivity of anticancer metallo-drugs with transferrin, allowing for the determination of the specific site to which the metallo-drug binds.25 Analyzing metal–protein complexes in physiological conditions requires the sample to be in a buffered solution. Buffer solutions are typically high in salt content and suppress ionization of the desired product,26 so to analyze the sample it must be in an ESI-compatible solution, such as acetonitrile. This will change the pH and ionic strength of the sample media, both of which may cause the metals to be released from complexes. Ideally, a technique that allows analysis of the metal–protein complexes without changing the sample media and for detection of non-metals and metals simultaneously would provide a more comprehensive technique for determining metal–protein interactions.

The particle beam/hollow cathode-optical emission spectrometer (PB/HC-OES), operates at inert atmosphere, utilizing glow discharge phenomena which allows detection of metals (i.e. Fe, Cr, Ni, or Ag) and non-metals (i.e. H, C, N, S, and O) simultaneously.7 The use of the particle beam allows for metal–protein complexes to be introduced from the original sample media by removing all solvent vapors and leaving only the dry analyte particle of interest to enter the hollow cathode source. Once particles enter the heated hollow cathode source (consisting of the glow discharge plasma) they are vaporized, atomized, and excited. The excited analyte atoms emit photons that are detected using a high resolution, vacuum polychromator, allowing for the determination of non-metal and metal species’ emission responses simultaneously. Recently, this laboratory has demonstrated the use of this instrument for determinations of loading percentages of Fe3+, Ni2+, Zn2+, and Ag+ bound to transferrin individually and as a competition study using M++(i)/C(i) ratios to determine empirical formulas that represent the loading percentages of each metal bound to transferrin.7 In addition, detection limits are in the low nM to μM range which allows for the analysis of metals that may have weak binding constants to transferrin with good precision and accuracy. Presented in this study is a comparison of UV-VIS absorbance, ICP-OES, and PB/HC-OES methods for investigating the interactions of Cr3+ with human transferrin to determine loading characteristics. Evaluation of the aforementioned methods will be conducted by studying chromium uptake at equilibrium conditions, kinetic aspects, varying companion anions, and under competition with iron for uptake into apo- and holo-transferrin.

Experimental

Sample preparation

High-purity (18.2 MΩ-cm) Barnstead Nanopure (Dubuque, IA) water and methanol (EMD Chemicals, Cincinnati, OH) were used as the primary solvents. Samples were prepared in 15 mL centrifuge tubes that had been washed with 1 M HCl, thoroughly rinsed, and dried to remove residual metals. Stock solutions of human holo- and apo-transferrin (50 μM, Sigma-Aldrich, St. Louis, MO) were prepared separately in Tris buffer (20 mM, TEKnova, Hollister, CA) at pH 7.4. Sodium carbonate (20 mM, Sigma-Aldrich) was added to all Tf solutions unless otherwise noted. Iron solutions were prepared by adding iron nitrate (45 μM, Sigma-Aldrich) and nitri-loacetic acid (NTA, 90 μM, Sigma-Aldrich) in a 1:2 ratio respectively to hydrochloric acid (0.1 M, J. T. Baker, Phillipsburg, NJ) and adjusting the pH to 4.0.27 Sodium citrate (1000 μM, Sigma-Aldrich) solutions were prepared in nanopure water. Chromium solutions were prepared by adding chromium nitrate or chromium chloride (1000 μM, Sigma-Aldrich) to nanopure water. Transferrin was loaded with chromium by adding 0–10 molar equivalents of Cr(NO3 )3 to the apo-transferrin solution, followed by incubation of the samples at 37 °C for 24 h.7 Following the incubation period the unbound metal was separated from the metal-loaded transferrin using a desalting Sephadex™ G-25 M PD-10 column (GE Healthcare, Buckinghamshire, UK).7 Since the chromium bound transferrin is colorless the desalting separation was done in a methodical way and repeated for all samples to ensure that all unbound chromium was separated prior to analysis. The procedure previously employed for the loading of the iron in transferrin,7 was shown to be quite robust and was incorporated here. In that method, the sample (2 mL of M++-Tf) was added to the desalting column, followed by 1 mL of tris buffer, and then an additional 2 mL of tris buffer was added and the 2 mL fraction was collected. After obtaining the sample fraction, re-equilibration of the column was done with 3 mL of tris buffer prior to the next usage.

pH Measurements

All solutions were pH measured with an Accumet Research AR 10 pH meter (Fisher Scientific, Pittsburgh, PA) with an
Accumet double junction Ag/AgCl pH probe (Fisher Scientific). Adjustments to pH were made with hydrochloric acid (6.0 M, Sigma-Aldrich) and sodium hydroxide (2.0 M, Sigma-Aldrich).

**Kinetic study**

10 molar equivalents of Cr(NO$_3$)$_3$ were added to a stock solution of apo-transferrin, followed by incubation at 37 °C. 2 mL aliquots were removed at 1, 3, 7, and 10 d (desalting took place immediately following incubation period prior to analysis).

**Anion and ligand study**

10 molar equivalents of Cr(NO$_3$)$_3$ and CrCl$_3$ with and without excess sodium carbonate (excess = 20 mM) and sodium citrate (1 mM) were added to the apo-transferrin solution, followed by incubation at 37 °C of the samples for 24 h (desalting took place immediately following incubation period prior to analysis).

**Fe$^{3+}$ vs. Cr$^{3+}$ Apo-transferrin competition study**

0–10 molar equivalent ratios of Fe(NTA)$_2$ to Cr(NO$_3$)$_3$ were added to the apo-transferrin solution, followed by incubation at 37 °C of the samples for 24 h (desalting took place immediately following incubation period prior to analysis).

**Displacement study**

0–10 molar equivalents of Cr(NO$_3$)$_3$ were added to the holo-transferrin solution, followed by incubation at 37 °C of the samples for 24 h (desalting took place immediately following incubation period prior to analysis).

All of the samples from each study above were then analyzed by ICP-OES, PB/HC-OES, and UV-VIS absorbance. All samples were kept in buffered solutions (pH 7.4) throughout the experiment so that no species-interconversion should have occurred during the metal loading process. 7

**UV-VIS Absorbance**

All absorbance measurements were performed with a Genesys 10-S UV-VIS spectrometer (Thermo Electron Corporation, Waltham, MA). The concentration of Tf was determined by measuring the absorbance at 280 nm and using an extinction coefficient of 87 200 M$^{-1}$ cm$^{-1}$. The concentration of Fe$^{3+}$ loaded into the Tf was determined by measuring the absorbance at 470 nm and using an extinction coefficient of 2400 grooves mm$^{-1}$ and a single photomultiplier tube as the detector. Data acquisition and instrument control were obtained by the JY Analyst v5.2 software. Operating parameters: power = 1000 W, Ar gas flow rate = 12.0 L min$^{-1}$, nebulizer = 0.02 L min$^{-1}$ at 1.0 bar, sheath gas flow rate = 0.20 L min$^{-1}$, peristaltic pump speed = 20.0 rpm, Fe(i) 259.94 nm, Cr(i) 283.56 nm.

**PB/HC-OES**

A particle beam/hollow cathode-optical emission spectrometer was used to determine metal and non-metal concentrations of the metal-loaded Tf solutions simultaneously as previously described. The PB/HC-OES instrument is a home-built system that has been through 15 years of improvements and optimization. The particle beam consists of a thermoconcentric nebulizer, desolvation chamber, and a two stage momentum separator. The nebulizer creates an aerosol spray that enters the desolvation chamber where the particles are further desolvated before passing through a two stage momentum separator that removes all low mass particles (e.g. solvent and nebulizer gas). After passing through the momentum separator, dry analyte particles enter into the copper hollow cathode glow discharge source where the particles are vaporized, atomized, and excited. A 0.5-m Paschen-Runge polychromator (JY RF-5000) consists of an ion-etched holographic grating (2400 grooves mm$^{-1}$) with a practical resolution of ~0.01 mm utilizing 26 photomultiplier tubes to detect the emission signals. The optical path was nitrogen-purged to allow for detection across the spectral range of 110–620 nm. The operating parameters: mobile phase = 50:50 water:methanol, nebulizer gas flow rate = ~1400 mL min$^{-1}$ He, nebulizer tip T = 105 °C, HPLC flow rate = 1.0 mL min$^{-1}$, 200 μL injection loop, desolvation chamber T = 150 °C, hollow cathode block T = 210 °C, discharge current = 60 mA, discharge pressure = 2 Torr He, C(i) 156.14 nm, Cr(i) 425.43 nm, Fe(i) 371.99 nm.

**Analytical responses**

ICP-OES calibration curves of 0.1–100 μM were prepared from stock standard solutions of Fe$^{3+}$ and Cr$^{3+}$ (High Purity Standards, Charleston, SC). PB/HC-OES calibration curves were prepared from aqueous standards of 0.1–500 μM Fe$^{3+}$ and Cr$^{3+}$, it has been previously determined that responses for metal ions are the same regardless of the sample matrix, allowing for quantitative analysis of the metal in both organic and inorganic forms. Typical response functions (derived from triplicate measurements from continuous sample introduction) for the ICP-OES system and response functions (derived from triplicate 200 μL injections at each concentration) for the PB/HC-OES system are presented in Table 1.

**Loading percentages**

All loading percentages are calculated based on the assumption that 2 molar equivalents of metal ions can be loaded into transferrin, meaning a 2 Fe : 1 Tf molar ratio would equate to 100% loading.
Results and discussion

Concentration effects on chromium loading

Previous work determining Cr\(^{3+}\)-Tf complexes using UV-VIS absorbance reported spectral bands at 293, 440, and 615 nm.\(^{44}\) Fig. 1a displays the spectral response of increased amounts of chromium loaded into apo-transferrin over a range of 225–650 nm. The spectra show spectral bands at 254, 298, and 428 nm that correspond to LMCT of tyrosine to the metal ion (chromium in this case), each showing increased absorbance values with increasing Cr\(^{3+}\) exposure. The weak band at 428 nm is specific to the chromium ion, while the two bands at 254 and 298 nm are the strongest, but are non-specific and represent the LMCT of any bound metal ion. For iron, spectral bands are found at 254, 298, and 470 nm that represent iron being loaded into the binding lobes of transferrin.\(^{21}\) These bands all show an increase that is proportional to the increasing amount of chromium available to bind to apo-transferrin. Fig. 1b represents the corresponding loading percentages of Cr\(^{3+}\) bound to apo-transferrin determined by both the ICP-OES and PB/HC-OES methods. The loading percentages in Table 2, found by both methods are in good agreement and are not significantly different at any value based on \(p\) values (\(<0.05\)). The loading percentages for the ICP method were determined with the use of the UV-VIS spectrophotometer (\(i.e.\) Cr concentration via ICP and Tf concentration via UV-VIS). The loading percentages for the PB/HC-OES method are reflective of the directly-measured ratio of Cr(i)/C(i).\(^{7}\) The maximum loading at a 10 molar equivalent excess was determined to be 26.8 ± 3.5% and 25.3 ± 2.2% for the ICP-OES and PB/HC-OES methods, respectively. This loading percentage was below expectations and should be much higher based on the stability constants of Cr\(^{3+}\) (\(K_{1} = 17\), estimated) and Fe\(^{3+}\) (\(K_{1} = 21\), measured).\(^{10}\) Previously, Fe\(^{3+}\) was found to load into transferrin at 71.2 ± 4.7% using this same method.\(^{7}\) Based on the aforementioned iron loading percentage and stability constant, it was expected that chromium would load into approximately 50% of the available apo-transferrin. A reasonable explanation to the lower than expected loading values is the fact that Cr\(^{3+}\) is an inert metal ion while Fe\(^{3+}\) is labile, so longer incubation times (than the 24 h used here) may be needed to fully load Cr\(^{3+}\) into transferrin.

Kinetic aspects of chromium loading

In order to determine if kinetics are limiting the exchange of Cr\(^{3+}\) from the solution environment to transferrin, excess sodium carbonate and a 10 molar equivalent excess of Cr\(^{3+}\) was added to a stock solution of apo-transferrin and allowed to incubate for 10 days. Fig. 2 represents the UV-VIS spectra ranging from 225–650 nm for aliquots removed from the stock solution ranging from day 1 to day 10. As can be seen from the spectra, the absorbance bands observed at 254 and 298 nm increase as the sample undergoes longer incubation times. The background increases with each sample over the range of 300–650 nm, making it impossible to quantify the amount of Cr\(^{3+}\) loaded into apo-transferrin using the 428 nm spectral band. Analysis of these chromium-transferrin complexes by the atomic spectroscopy methods confirms that changes in the absorbance spectra at 428 nm do not coincide with the actual amount of Cr\(^{3+}\) loaded into apo-transferrin. The data obtained from both instruments were in good agreement for all but the day 1 sample (\(p\) value <0.05). Table 3 shows after 10 days of incubation the loading percentages increased to...

### Table 1: Typical elemental quantification data for PB/HC-OES and ICP-OES methods

<table>
<thead>
<tr>
<th>Element</th>
<th>Response function</th>
<th>Accuracy ((R^2))</th>
<th>LODs/μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB/HC-OES</td>
<td>Fe(i)</td>
<td>(y = 0.0016 \times -0.0018)</td>
<td>0.9912</td>
</tr>
<tr>
<td></td>
<td>Cr(i)</td>
<td>(y = 0.0397 \times -0.1470)</td>
<td>0.9899</td>
</tr>
<tr>
<td></td>
<td>C(i)</td>
<td>(y = 0.0047 \times -0.0082)</td>
<td>0.9694</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Fe(i)</td>
<td>(y = 7819.7 \times +1421.9)</td>
<td>0.9997</td>
</tr>
<tr>
<td></td>
<td>Cr(i)</td>
<td>(y = 0.0397 \times -0.1470)</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Fig. 1 Additions of 0–10 molar equivalents of Cr\(^{3+}\) loaded into apo-transferrin. (a) UV-VIS spectrum and (b) loading percentages determined by PB/HC-OES method from Cr\(^{3+}/C\) ratios and ICP-OES method with UV-VIS absorbance jointly to determine metal and protein concentrations for loading percentages.
49.7 ± 4.5% and 55.7 ± 3.2% transferrin loaded with Cr\(^{3+}\) as determined by the ICP-OES and PB/HC-OES methods, respectively. A loading value of ∼55% transferrin loaded with Cr\(^{3+}\) is closer to expectations based on the estimated stability constant for a Cr\(^{3+}\)-Tf complex. Based on these results, it is necessary for Cr\(^{3+}\) to be in the presence of transferrin for an extended period of time (up to 10 days) to get maximum loading.

**Anion ligand effects on chromium loading**

Carbonate is a synergistic anion that is important for the binding of metal ions into the N- and C-terminus lobes of transferrin; without carbonate, metal-transferrin binding is weak.\(^5\) Citrate is a chelating agent that is commonly used to load Cr\(^{3+}\) (i.e. chromium-citrate) into apo-transferrin in vitro, with \(pK_a\) (2.9, 4.3, and 5.6) all well below the physiological pH 7.4.\(^5\) The presence of citrate and carbonate in solution with Cr\(^{3+}\) plays a role in the loading of chromium into apo-transferrin. Table 4 represents the loading percentages of transferrin bound with Cr\(^{3+}\) under various loading conditions with and without excess citrate and carbonate.

The UV-VIS spectra (not presented here) of chromium nitrate and chromium chloride do not reflect the actual amount of Cr\(^{3+}\) that has been loaded into apo-transferrin. As presented above, the higher amounts of Cr\(^{3+}\) loaded into apo-transferrin result in an increased background that interferes with the 428 nm band so the study was monitored using the ICP-OES and PB/HC-OES methods.

As demonstrated by the data presented in Table 4, the addition of anions limit the uptake of Cr\(^{3+}\) into transferrin as a larger amount of chromium was loaded into apo-transferrin when there was no citrate present. The loading percentages suggest that citrate is competing with transferrin for Cr\(^{3+}\) binding during the 24 h incubation time. When comparing the nitrate versus chloride Cr\(^{3+}\) species, it would seem that the larger loading percentages observed for nitrate are due to it acting as a better synergistic anion than chloride when there is no excess carbonate present. Another possibility is the fact that chloride plays an important part in the release of iron from transferrin,\(^46\) so the excess chloride may promote some loss of Cr\(^{3+}\) from the transferrin binding pocket. Once carbonate is introduced into the loading environment, the loading percentages are cut almost in half as a competition between carbonate and transferrin for chromium may be occurring in solution. Under these experimental conditions, the inertness of Cr\(^{3+}\) combined with the presence of carbonate results in lower binding percentages of Cr\(^{3+}\) into apo-transferrin (i.e., with preference to carbonate). Bertini and co-workers studied the effects of chloride and carbonate on copper-transferrin complexes, presenting results showing that increased amounts of chloride will cause copper to be released from transferrin and that increased amounts of carbonate could cause the reloading of copper into transferrin.\(^47\)

The results of the above experiments show that chromium binds into apo-transferrin more efficiently when citrate and carbonate are not present in excess. Under physiological conditions carbonate will always be present at 20 mM, thus this concentration of carbonate was used for the remainder of the studies. It was also revealed that it takes >24 h to get maximum loading amounts of Cr\(^{3+}\) into apo-transferrin. The

---

**Table 2** Cr\(^{3+}\) loading into apo-Tf, incubated @ 37 \(^{\circ}\)C for 24 h

<table>
<thead>
<tr>
<th>Molar equivalent additions of Cr(^{3+})</th>
<th>ICP-OES</th>
<th>PB/HC-OES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Tf Loaded with Cr(^{3+})</td>
<td>Cr(^{3+})/C Ratio</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.220</td>
</tr>
<tr>
<td>2</td>
<td>12.6 ± 1.8</td>
<td>0.242</td>
</tr>
<tr>
<td>6</td>
<td>20.5 ± 2.8</td>
<td>0.248</td>
</tr>
<tr>
<td>8</td>
<td>21.9 ± 2.4</td>
<td>0.253</td>
</tr>
<tr>
<td>10</td>
<td>26.8 ± 3.5</td>
<td>0.268</td>
</tr>
</tbody>
</table>

---

**Table 3** Kinetic study of Cr\(^{3+}\) loading into apo-Tf

<table>
<thead>
<tr>
<th>10 Molar equivalents of Cr(^{3+}) incubated for</th>
<th>ICP-OES</th>
<th>PB/HC-OES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Tf Loaded with Cr(^{3+})</td>
<td>Cr(^{3+})/C Ratio</td>
</tr>
<tr>
<td>1 Day</td>
<td>35.5 ± 3.5</td>
<td>0.280</td>
</tr>
<tr>
<td>3 Days</td>
<td>37.7 ± 3.9</td>
<td>0.308</td>
</tr>
<tr>
<td>7 Days</td>
<td>47.8 ± 3.3</td>
<td>0.345</td>
</tr>
<tr>
<td>10 Days</td>
<td>49.7 ± 4.5</td>
<td>0.383</td>
</tr>
</tbody>
</table>
kidneys remove most substances in approximately 24 h, however the half life for chromium varies from 4 to 35 h. Therefore, the remainder of the studies presented here were kept at 24 h to allow for closer approximations to equilibrium conditions and comparisons to other loading experiments. It was also deemed unnecessary to use citrate for the loading procedure, since there was suppression in loading observed with citrate. Additionally, serum concentrations of citrate would be 10^9 lower in vivo (~100 μM).

Competitive binding of Cr^{3+} and Fe^{3+} into apo-transferrin

To understand the effects of Cr^{3+} and Fe^{3+} binding into apo-transferrin under both low metal and excess metal conditions, various iron to chromium ratios were introduced into the test media. Fig. 3 represents the UV-VIS absorbance spectra of the competition between iron and chromium loading into apo-transferrin. Bands at 254 and 298 nm, which are non-specific to the identity of the metal loaded into transferrin, increase representing increased total metal ions bound to the tyrosine residues in the binding lobes. More importantly for these studies, there is an overlap of the bands at 428 and 470 nm for Cr^{3+} and Fe^{3+}, respectively, that makes determining the amount of each individual metal very difficult. Fig. 4a and b graphically display the results of the competitive binding experiments for the two metal ions (Fe^{3+} and Cr^{3+}, respectively) as determined by ICP-OES (in conjunction with UV-VIS absorbance), PB/HC-OES, and UV-VIS absorbance. Fig. 4a displays the amount of Fe^{3+} loaded into apo-transferrin determined by all three methods.

Table 4  Anion and ligand effects on Cr^{3+} loading into apo-Tf, incubated @ 37 °C for 24 h

<table>
<thead>
<tr>
<th>10 Molar Equivalent Additions</th>
<th>ICP-OES</th>
<th>PB/HC-OES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Tf Loaded with Cr^{3+}</td>
<td>Cr^{3+}/C Ratio</td>
</tr>
<tr>
<td>Cr(NO_3)_3</td>
<td>71.0 ± 3.1</td>
<td>0.437</td>
</tr>
<tr>
<td>Cr(NO_3)_3 + citrate</td>
<td>57.2 ± 2.5</td>
<td>0.391</td>
</tr>
<tr>
<td>Cr(NO_3)_3 + citrate + CO_3^-</td>
<td>31.0 ± 3.7</td>
<td>0.293</td>
</tr>
<tr>
<td>CrCl_3</td>
<td>28.8 ± 4.1</td>
<td>0.275</td>
</tr>
<tr>
<td>CrCl_3 + citrate</td>
<td>60.7 ± 2.1</td>
<td>0.411</td>
</tr>
<tr>
<td>CrCl_3 + citrate + CO_3^-</td>
<td>52.6 ± 3.3</td>
<td>0.373</td>
</tr>
<tr>
<td>CrCl_3 + CO_3^-</td>
<td>35.5 ± 2.1</td>
<td>0.315</td>
</tr>
<tr>
<td>CrCl_3 + CO_3^-</td>
<td>34.7 ± 3.9</td>
<td>0.304</td>
</tr>
</tbody>
</table>

Fig. 4  (a) Displays a comparison of the Fe^{3+} loading percentages by UV-VIS, ICP-OES, and PB/HC-OES methods. * denotes a significant difference between the ICP-OES percentage and the UV-VIS absorbance percentage determined by p value < 0.05. (b) Displays a comparison of the Cr^{3+} loading percentages by ICP-OES and PB/HC-OES methods. * denotes a significant difference from the first data points (0 Fe : 2 Cr) determined by p value < 0.05.

A noticeable difference in the amount of Fe^{3+} bound to transferrin can be seen for the situations where excess metal was used for the loading study. These discrepancies are most likely attributed to the spectral interferences and overlap of the two absorption bands for iron and chromium. Therefore, UV-VIS cannot be used by itself for determining the amount of iron bound when multiple metals are available for loading into apo-transferrin.

Fig. 4b represents the amount of Cr^{3+} that was loaded into apo-transferrin under the competitive conditions. Note first
that UV-VIS in and of itself is not applicable for Cr\(^{3+}\) loading
determinations. Looking at the loading trends from the
ICP-OES and PB/HC-OES methods, displayed Fig. 4a and
b, there is no competition between Cr\(^{3+}\) and Fe\(^{3+}\) when added
at a 2:2 molar equivalent ratio (compared to the values
of each metal ion separately). However, when the molar
equivalent ratio of iron is 5× that of chromium (10:2 ratio),
the iron loading is 5.75 times that of chromium. In order to get
equal amounts of Fe\(^{3+}\) to Cr\(^{3+}\) loaded into apo-transferrin,
5 times the amount of chromium to iron is required. When
both metals are introduced to the apo-transferrin at excess
(10:10 ratio), iron loads into apo-transferrin at a ratio
2.58 times greater than chromium. There are many reports
suggesting that the presence of iron reduces the amount of
chromium loading into transferrin and report a 1 Fe : 1 Cr
loading ratio,\(^{44,50–53}\) but these reports lack quantitative data to
support this.

In summary the data suggests that in the case of excess Cr\(^{3+}\)
as (might occur in cases of chromium toxicity), apo-transferrin
bonds up to 30% iron meaning that cells should still
uptake close to the normal amount of iron.\(^{5}\) According to
Vincent, there may be a direct link between diabetes and
hemachromatosis.\(^{15}\) The data presented here reveals that
chromium loading decreases under excess iron conditions
(hemochromatosis patients), suggesting that hemochromatosis
leads to lower chromium levels in the body. It is clear from
the data that regardless of the amount of Cr exposure (up to
10× stoichiometry), the iron loading percentages always
remain at or above the physiological values found in the body.

**Competition of Cr\(^{3+}\) with bound iron in holo-transferrin**

To further understand how Cr\(^{3+}\) affects Fe\(^{3+}\) loading into
transferrin, molar equivalent additions of Cr\(^{3+}\) were added to
holo-transferrin (~90% iron loaded, 1.8 Fe : 1 Tf). The pH
was monitored and maintained at 7.4 throughout the studies
to assure that a change to more acidic conditions was not the
cause of iron release. Although not shown here, the UV-VIS
spectrum for molar additions of Cr\(^{3+}\) added to holo-transferrin
reveals a decrease in Fe\(^{3+}\) loading due to the decrease in
absorbance seen at 470 nm. As seen for the previous data, it
is impossible to determine whether or not Cr\(^{3+}\) was loaded into
the transferrin binding pockets after the iron was displaced using
the UV-VIS absorbance spectrum alone. In order to determine
the loading percentages for both metal ions, ICP-OES and
PB/HC-OES methods were used. The data presented in Fig. 5
reflect a scenario wherein Cr\(^{3+}\) displaces the iron and is then
loaded into the now empty binding pocket of transferrin. This is
consistent with reports in the literature of decreased iron loading
due to Cr\(^{3+}\) presence in the loading environment.\(^{44,50–53}\)

**Conclusion**

The data acquired from the PB/HC-OES method matches the
data obtained from the ICP-OES method in combination with
the UV-VIS spectrophotometer. This further supports the use
of the PB/HC-OES method as a potential metallomics tool
for studying metal binding proteins. The uniqueness of this
method allows acquisition of metal/protein ratios that are not
affected by recovery losses and are a direct representation of
the metal–protein complex. Spectral interference issues
and overlapping LMCT absorbance bands with the UV-VIS
spectra have been displayed for scenarios involving
Cr\(^{3+}\)-transferrin complexes and multiple metals loaded into
apo-transferrin. It must be made clear that UV-VIS absorbance
is still a very useful technique and must be used jointly with the
ICP-OES method. It has also been demonstrated that the
PB/HC-OES method provides information that is normally
collected from multiple instruments on a single platform.

Results of these studies provide new insight into
Cr\(^{3+}\)/Fe\(^{3+}\)-transferrin chemistry. Due to the inert nature,
loading of Cr\(^{3+}\) into apo-transferrin is much slower than for
Fe\(^{3+}\) and requires up to 10 days to achieve 50% loading.
In order to get loading values similar to that of Fe\(^{3+}\) (~72% Fe
loading reported previously with this same PB/HC-OES
method) non-physiological conditions were required. Although
the presence of Cr\(^{3+}\) caused some inhibition of the ability for
Fe\(^{3+}\) to be loaded into transferrin, 5 times the amount of
chromium to iron was required to get an equal loading scenario
(30% Fe : 30% Cr). This potentially means that even at ratios of
1 Fe : 0.8 Cr as might be seen with chromium toxicity, iron is
still delivered into the cell. For the case where iron is in excess of
up to 5 times that of Cr\(^{3+}\) there is a slight inhibition in the
ability for Cr\(^{3+}\) to be loaded into apo-transferrin. In addition,
holo-transferrin was observed to release Fe\(^{3+}\) in the presence of
increasing amounts of Cr\(^{3+}\).

**Acknowledgements**

Thanks to Dr K. Christensen at Clemson University for
allowing use of the Genesys 10-S UV-VIS spectrometer.

**References**
