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RESEARCH ARTICLE

Evaluation of Gallium as a Tracer of Exogenous Hemoglobin–Haptoglobin Complexes for Targeted Drug Delivery Applications

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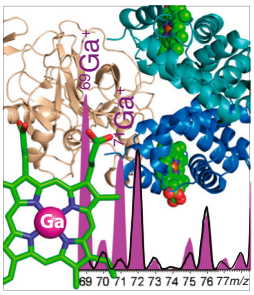

Abstract. Haptoglobin (Hp) is a plasma glycoprotein that generates significant interest in the drug delivery community because of its potential for delivery of antiretroviral medicines with high selectivity to macrophages and monocytes, the latent reservoirs of human immunodeficiency virus. As is the case with other therapies that exploit transport networks for targeted drug delivery, the success of the design and optimization of Hp-based therapies will critically depend on the ability to accurately localize and quantitate Hp-drug conjugates on the varying and unpredictable background of endogenous proteins having identical structure. In this work, we introduce a new strategy for detecting and quantitating exogenous Hp and Hp-based drugs with high sensitivity in complex biological samples using gallium as a tracer of this protein and inductively coupled plasma mass spectrometry (ICP MS) as a method of detection. Metal label is introduced by reconstituting hemoglobin (Hb) with gallium(III)-protoporphyrin IX followed by its complexation with Hp. Formation of the Hp/Hb assembly and its stability are evaluated with native electrospray ionization mass spectrometry. Both stable isotopes of Ga give rise to an abundant signal in ICP MS of a human plasma sample spiked with the metal-labeled Hp/Hb complex. The metal label signal exceeds the spectral interferences' contributions by more than an order of magnitude even with the concentration of the exogenous protein below 10 nM, the level that is more than adequate for the planned pharmacokinetic studies of Hp-based therapeutics.

Keywords: Protein therapeutics, Protein labeling, Metal tags, Noncovalent complexes, Native electrospray ionization mass spectrometry, Human immunodeficiency virus, Targeted drug delivery

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Introduction

Haptoglobin (Hp) is an acute phase plasma protein, the *raison d'être* of which is sequestration of free hemoglobin (Hb) in circulation to avoid possible renal damage and other negative physiological consequences of intravascular hemolysis [1]. In the past, interest in Hp was caused primarily by its obvious ability to attenuate efficiency of oxygen transport by Hb-based blood substitutes [2], as well as its involvement in iron acquisition by several pathogens [3]. More recently, better understanding of the Hp-mediated pathway of Hb clearance [4, 5] has provided strong indications that this protein may also be used for targeted drug delivery, taking advantage of the fact that Hb · Hp complexes are processed (catabolized) in macrophages [6, 7]. Since macrophages and their progenitors (monocytes) play a prominent role in establishing certain types of viral infections (including human immunodeficiency virus, HIV), virus dissemination, and development of viral reservoirs [8], the ability to deliver anti-viral therapeutics directly to macrophages (e.g., by conjugating them to either Hp or Hb · Hp complexes) should result in a dramatic improvement of the drug efficacy. Another high value target for such a strategy might be the hepatitis C virus (HCV), since there is evidence that resident liver macrophages are infected by and support replication of HCV [9]. In addition to viral infections, a similar strategy can be envisioned as a way to design novel therapies against certain types of cancers (most notably acute myeloid leukemia), as the monocyte/macrophage lineage specificity of CD163 (cell-surface receptor that recognizes Hb · Hp complexes and assists their internalization [7, 10]) expression is preserved beyond malignant transformation [11, 12]. The specificity of targeted cytotoxin delivery in this case would be

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particularly beneficial, as Hp-mediated drug delivery specifically to the CD163-expressing cells will be limited to monocytes and macrophages and spare normal stem cells in the bone marrow [11].

As is the case with all drug delivery strategies utilizing transport proteins for targeted delivery, successful design of therapies based on Hp as a delivery vehicle (e.g., Hp-cytotoxin or Hp-antiviral drug conjugates) will critically depend on the ability to trace the drug/protein conjugate within the organism following its administration to obtain detailed and reliable pharmacokinetics and pharmacodynamics information [13–15]. While the protein quantitation strategies are now well-established, a unique challenge for reliable and sensitive localization and quantitation of protein therapeutics based on or mimicking abundant endogenous proteins (such as Hp) arises from the fact that such measurements are carried out on the varying (and often unpredictable) background of endogenous molecules, the structure of which is identical to that of the exogenous (administered) proteins. This makes it highly problematic to use classic techniques of protein quantitation, such as enzyme-linked immunosorbent assay (ELISA) [16, 17], which remains one of the most popular and sensitive techniques in the field [18, 19]. Recently, mass spectrometry (MS) emerged as a powerful tool for protein quantitation in complex biological matrices, enabling highly sensitive and selective measurements both in the field of proteomics [20] and in a variety of pharmacokinetic studies [21–23]. Most of these applications rely on stable isotope labeling of proteins or their fragments as a means of quantitation [24]. However, because most cost-effective labeling strategies introduce the isotopic label during the proteolytic step [25], they frequently fail to make a distinction between an exogenous protein and its endogenous counterpart if the degree of structural similarity between them is very high. Such a distinction can be made if the isotopic label is introduced at the whole protein level (e.g., during the expression of the exogenous protein), but this usually results in a dramatic increase of the production costs.

An alternative approach to introducing distinct labels to exogenous proteins that can be readily identified by MS takes advantage of the possibility to attach a metal tag to the protein surface, making it detectable by inductively coupled plasma (ICP) MS [26]. A very important advantage of this approach is the high sensitivity afforded by ICP MS measurements and the possibility to select a non-cognate metal tag that would have minimal spectral interferences. However, an obvious drawback of these strategies is their reliance on chemical modification of the protein surface, which may alter its properties, including interactions with physiological partners and therapeutic targets. Recently, we introduced a solution to this problem that can be used when the exogenous protein is a metalloprotein (e.g., ferro-protein transferrin used to deliver drugs either to malignant cells or across physiological barriers [27]), where the cognate metal is replaced with a non-cognate one without altering protein conformation or compromising the receptor recognition. For example, replacing Fe^{3+} with In^{3+} in transferrin allows this protein to be detected with high sensitivity in blood and tissue homogenates of animals using ICP MS, and its distribution maps to be obtained by imaging with laser ablation ICP MS [28].

In this work, we extend this approach to Hp, which is not a metalloprotein. This is done by substituting Fe^{3+}-bound protoporphyrin IX (heme) with the protoporphyrin IX molecule that contains a non-cognate metal (gallium) within Hp's counterpart, Hb prior to forming the Hp·Hb complex (which is the molecular entity recognized by the cell-surface receptor). Despite relatively low stability of Ga-substituted Hb (Hb^{Ga}), it binds readily to Hp, with the resulting $Hp{\cdot}Hb^{Ga}$ complex remaining remarkably stable under physiological conditions. The non-cognate metal allows this complex to be readily detected in serum samples at levels well below 10 nM, which makes it suitable for pharmacokinetic studies.

Experimental

Materials

Hp phenotype 1-1 was purchased from Athens Research and Technology (Athens, GA, USA), and human Hb was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Gallium (III)-protoporphyrin IX (Ga-PP) was purchased from Frontier Scientific (Logan, UT, USA). Gallium, plasma standard solution (10,000 mg/L) was purchased from Alfa Aesar (Haverhill, MA, USA). Nitric acid (69.2% aqueous solution) and hydrogen peroxide (31.4% aqueous solution) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Human serum samples were provided by the Department of Kinesiology, University of Massachusetts-Amherst (Amherst, MA, USA). All other reagents and solvents used in this work were of analytical grade or higher.

Methods

The apo-form of human Hb was prepared using a modified acidic acetone precipitation method described in detail elsewhere [29]. Briefly, cold (4 °C) aqueous solution of 200 μM Hb in 150 mM ammonium acetate was infused into cold (0 °C) 2 M HCl acetone solution (mixing ratio 1:50), followed by vigorous mixing for 5 min. The fluffy, colorless precipitate was collected by pipette, centrifuged at 4 °C and lyophilized. The lyophilized apo-Hb was dissolved in 10 mM ammonium acetate solution to a final concentration of 0.42 mg/mL and placed on ice for at least 1 h, followed by the addition of 1 mM Ga-PP methanol solution at a 37:1 ratio (v:v). The molar ratio of Ga-PP to globin monomers was estimated to be 1:1. This solution was incubated on ice for 1 h to produce Hb^{Ga}, followed by ESI MS and/or UV/Vis absorption analyses at room temperature. The $Hp{\cdot}Hb^{Ga}$ complexes were produced by adding 44 μM solution of Hp in 150 mM ammonium acetate to the ice-cold Hb^{Ga} solution at a mixing ratio of 1:13 (v:v), corresponding to an 8:1 molar ratio of Hb^{Ga} globins (monomers) to Hp.

A Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure the UV-Vis

absorption spectra of Hb^{Ga} and $Hp{\cdot}Hb^{Ga}$ and evaluate their stability at 37 °C by monitoring the decay rates of their Soret bands within the time interval ranging from 0.5 to 24 h. The measured intensities of the Soret bands were fitted to exponential decay curves to estimate the half-life values. SEC fractionation of $Hp \cdot Hb^{Ga}$ was carried out using Agilent 1100 (Agilent, Santa Clara, CA, USA) liquid chromatograph equipped with a TSKgel G3000SWxl (Tosoh, Tokyo, Japan) column. A 150 mM ammonium acetate solution was used as mobile phase in the separation at a flow rate of 0.8 mL/min. The absorbance was measured at 280 and 415 nm. SEC fractions were manually collected and characterized by native electrospray ionization (ESI) MS with a QStar-XL (AB/Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer equipped with a nano-spray source.

Human serum spiked with $Hp{\cdot}Hb^{Ga}$ was prepared using the metal-labeled protein solution in which the total protein concentration was determined by UV absorption measurements at 280 nm using the molar absorptivity (165,700 $M^{-1} \cdot cm^{-1}$) calculated based on the sequence of both Hp and Hb. The detection of Ga in the spiked serum samples was carried out using NexION 300X (Perkin Elmer, Waltham, MA, USA) ICP mass spectrometer following its overnight digestion with 49% HNO_3 and 7% H_2O_2 (by volume) at 37 °C. The kinetic energy discrimination (KED) mode was used to reduce polyatomic interference. The ICP mass spectra were obtained for both the spiked serum sample and the blank (Ga-free serum) by scanning the quadrupole in the *m*/*z* range 50–150 *u*.

Results and Discussion

Rationale for Selecting Gallium as a Metal Tracer

Hp is not a metalloprotein, but its binding partner Hb is. Since their association is very strong [30], and the Hp · Hb complex is in fact the molecular entity recognized by CD163 on the macrophage surface (an obligatory first step in its internalization), Hb appears to be an excellent choice for introducing a metal tag that can be used to trace the entire complex. Although the cognate metal (iron) is obviously unfit to be a good tracer (due to its abundance in living organisms), substitution of this metal with a non-cognate one will produce a good tag as long as it does not disrupt (1) the heme interaction with each of the globin chains (both α and β), and (2) the association of Hp with the reconstituted Hb. There is a wide variety of nonferrous metals that can be complexed with protoporphyrin, some of them having physiological significance [31]. However, gallium appears to be the best substitute for the cognate metal due to the filled *d*-shell, very close size match (0.62 Å versus 0.65 Å for Fe^{3+}) and its trivalency (even though heme-bound iron exists in the ferrous state inside erythrocytes, hemolysis results in its rapid oxidation to Fe^{3+}, leading to formation of met-hemoglobin, which is the form interacting with Hp in circulation). In fact, Ga-substituted protoporphyrin (Ga-PP) is commonly considered to be a good model to study the heme–globin interaction [32]. Furthermore, gallium is not an essential metal and, unlike most of its neighbors in the periodic table, it is not present in the human body at levels exceeding 0.2 ppb [33] (unless the subjects had chronic exposure to this metal [34]), which should minimize interferences in ICP MS and LA-ICP MS measurements.

Production of Hb^{Ga} and Evaluation of Its Stability

A recent study by Pinter et al. demonstrated that Ga-PP can be readily incorporated in myoglobin, a protein that is highly homologous to the Hb α- and β-chains, although the Ga-PP reconstituted protein remained stable only at low temperatures [32]. Incorporation of Ga-PP into Hb in our work was also carried out at low temperature. The correct placement of the prosthetic group within the polypeptide chains was indirectly verified by UV-Vis absorption spectroscopy (Figure 1), which shows a significant red shift of the B-band of Ga-PP (from 406 to 415 nm), consistent with the formation of the Soret band; a very similar Ga-PP "insertion signature" was previously reported for myoglobin [32]. A more detailed examination of the composition of the products of Hb reconstitution with Ga-PP was carried out using native ESI MS, which revealed $(\alpha^{Ga}\beta^{Ga})_2$ as a species giving rise to the most abundant ionic signal (Figure 2). The general appearance of this mass spectrum is very close to that previously reported for commercial ferro-Hb, Hb^{Fe} [35, 36]. In addition to the tetrameric species, both dimers and monomers are observed, alongside some heme-deficient species (labeled with open circles in Figure 2). Some of these Ga-PP deficient species (i.e., ions lacking a prosthetic group) may be produced by gas-phase dissociation, as we note the presence of ions in the low *m*/*z* region of the mass spectrum, the masses and isotopic distribution of which allow them to be unequivocally identified as singly charged Ga-PP (see the inset in Figure 2). These ions are likely to be produced in the gas phase (heme loss is a very common fragmentation pathway for

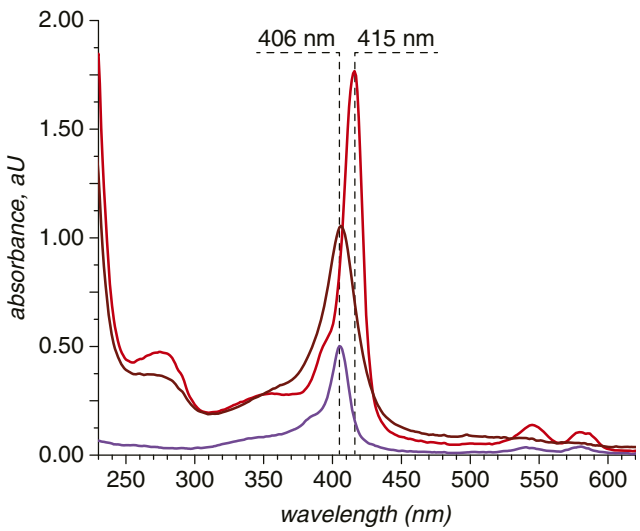

Figure 1. UV-Vis spectra of a water/methanol solution of Ga-PP (purple), and aqueous solutions of Hb^{Ga} (red) and Hb^{Fe} (brown)

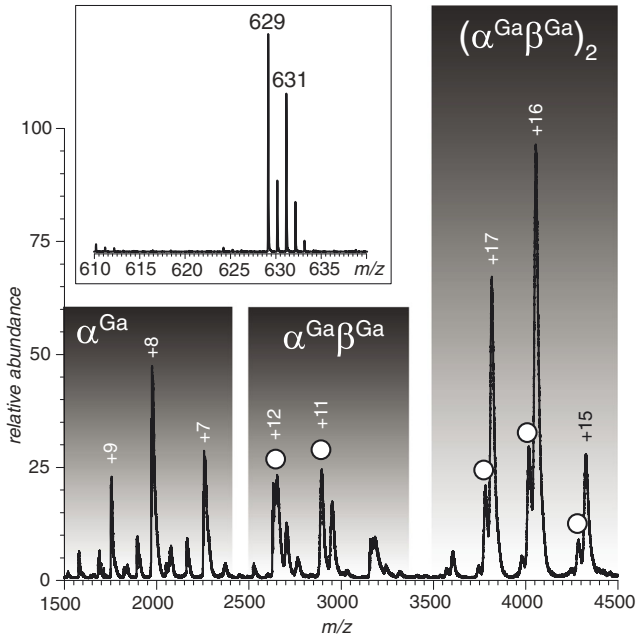

Figure 2. Native ESI mass spectrum of a 5 μM solution of Hb^{Ga} (human Hb reconstituted with Ga-PP) in 150 mM ammonium acetate acquired 60 min after the incubation. Open circles indicate hemoglobin species lacking one Ga-PP group (i.e., tetramers with three Ga-PP groups and dimers with a single Ga-PP group). The inset shows a low *m/z* range of the mass spectrum containing the ionic signal of Ga-PP

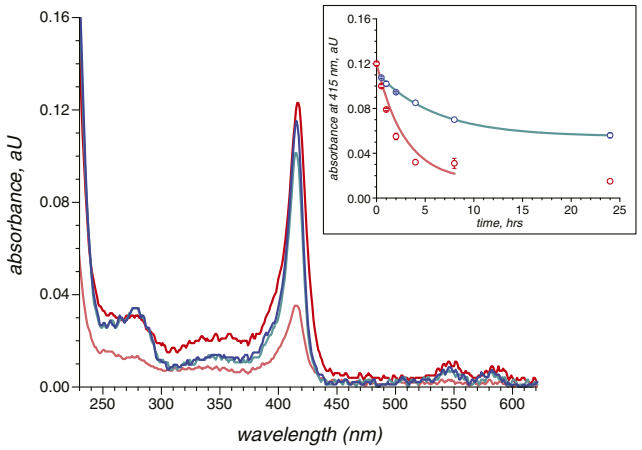

Figure 3. UV-Vis absorption spectra of Hb^{Ga} (red and pink) and Hp · Hb^{Ga} (blue and teal); the delay before the acquisition of the second spectrum in each pair was 2 h. The insert shows the evolution of the intensity of the Soret band in each sample over a 24-h time period

all globins), but it cannot be ruled out that at least some heme-deficient species are present in solution as well. Indeed, the presence of heme-deficient assemblies was previously observed even for Hb^{Fe} [35], and was ascribed to the presence of oxidized β-chains in the commercial Hb samples, which have diminished ability to retain the heme group [36] and significantly enhanced flexibility [37].

Unlike Hb^{Fe}, Hb^{Ga} was observed to precipitate even at 4 °C, which manifested itself by clouding of the protein solution, followed by formation of visible solid precipitates. One possible explanation for this increased aggregation propensity is that the negatively charged Ga-PP in solution may electrostatically repel the negative β-globin in which Cys^{93} (pK_a = 8.2) is oxidized to cysteine sulfinic acid (pK_a = 2) or sulfonic acid (pK_a = –3) [38]. This would exclude partial solvent for β-globins and expose hydrophobic residues, triggering nucleation and irreversible aggregation [39]. The rate of Hb^{Ga} loss in solution at physiologically relevant temperature (37 °C) was measured by monitoring the intensity of the Soret band using SEC-purified Hb^{Ga} as a starting material (Figure 3). A very dramatic decrease of the band intensity is observed within few hours (compare the red and pink traces in Figure 3 corresponding to measurements taken 2 h apart). The rate of the Soret band decay in Hb^{Ga} sample is consistent with the half-life of this protein being only 2 h, indicating its high vulnerability under conditions similar to those encountered in circulation.

Hp is known to exert a significant stabilizing effect on proteins it binds, and for that reason is frequently referred to as an "extracellular chaperon" [40, 41]. Since the therapeutic action of Hp/drug (or Hb/drug) conjugate could be exerted only within the context of Hp · Hb complex, possible stabilization of Hb^{Ga} by Hp would be very relevant vis-à-vis ensuring the reliability of the measurements by minimizing or indeed eliminating the loss of the protein due to aggregation. In order to evaluate the stability of Hb^{Ga} in complex with Hp, a mixture of the two proteins was incubated at 4 °C followed by SEC fractionation (Figure 4). Both SEC fractions exhibited absorbance at 415 nm, and were assigned as Hp · Hb^{Ga} (elution window 8.9–9.8 min) and free Hb^{Ga} (elution window 12.5–14 min); this assignment was confirmed by native ESI MS analysis of both fractions (vide infra). Monitoring the intensity of the Soret band for the early-eluting species (assigned as Hp · Hb^{Ga} complex) over a 1-day period following the fraction collection revealed a markedly increased stability compared to free Hb^{Ga}. In fact, the measured rate of the Soret band decay was consistent with the half-life of ca. 24 hours. This time window appears to be more than adequate for the measurements of biodistribution of Hp-based drugs targeting macrophages, as the physiological clearance of Hp · Hb occurs on a significantly shorter time scale [42].

Characterization of Hp·Hb^{Ga}

The SEC fractions of the Hb^{Ga}/Hp mixture (1.4 mg/mL and 0.27 mg/mL, respectively) incubated at 4 °C overnight were also analyzed by native ESI MS. The off-line ESI mass spectrum of the earlier eluting species (a fraction collected within the 8.9–9.8 min elution window) acquired under near-native condition (blue trace in Figure 4, bottom) contains contributions from several ionic species. The ionic signal of the major contributing species is confined to a narrow *m/z* window 5000–6000, and its mass is calculated as 157.7 kDa based on *m/z* values for the centroids of the three most abundant peaks. This mass is 2.7 kDa higher than the theoretical mass for the

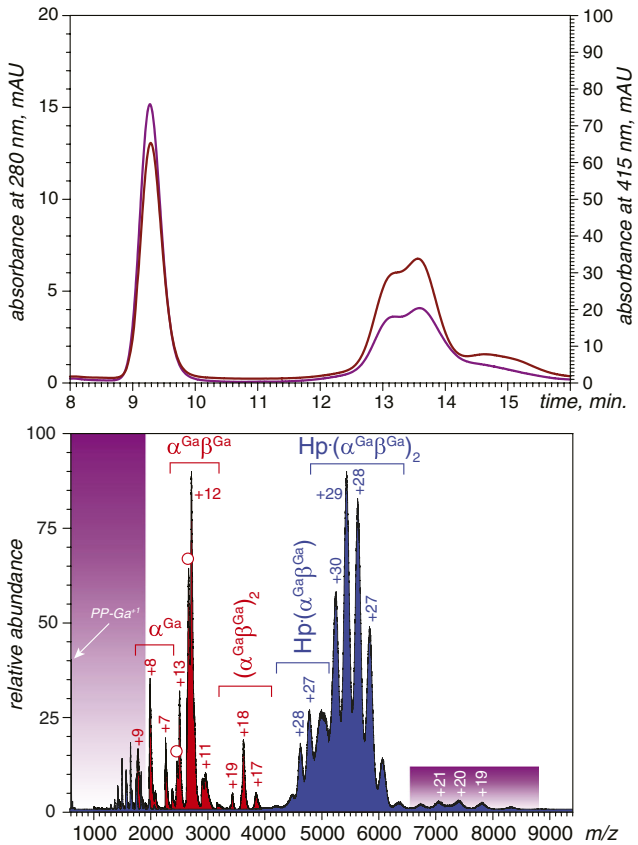

Figure 4. Top: size exclusion chromatogram of Hp and Hb^{Ga} mixture acquired at two different wavelengths (280 nm, purple, and 415 nm, brown). Bottom: off-line ESI mass spectra of SEC fractions acquired over 9–10 min (blue) and 13–14 min (red) time windows. The shaded areas in the mass spectrum of the early-eluting fraction contain gas-phase fragment ions produced via asymmetric charge partitioning. Open circles indicate hemoglobin dimers with a single Ga-PP group

$Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$ complex (calculated as 154,984.6 Da for the major glycoform of Hp and assuming no modification to the sequences of all proteins involved), and a mismatch of this magnitude is to be expected given the extensive solvation of large protein ions generated by native ESI [43, 44]. Indeed, increasing collisional activation of ions in the ESI interface region by stepping up the declustering potential resulted in a noticeable shift of these peaks towards lower *m/z* values (consistent with the notion of the partial loss of small molecules from the solvation shell of the surviving complex ion), although this also increased the efficiency of other dissociation channels (see Supplementary Material for more detail).

A cluster of low-abundance peaks at high *m/z* (>6000 *u*) corresponds to the products of gas-phase dissociation of $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$ ions proceeding via a loss of a single globin chain (the complementary fragments populate the low *m/z* region of the mass spectrum (<2000 *u*). This dissociation channel (asymmetric charge partitioning, where a single highly charged polypeptide chain is ejected from the complex ion), is usually a preferred dissociation channel of protein complexes in the gas phase [45, 46]. Lastly, a group of lower-abundance ions populate a narrow *m/z* region of the mass spectrum (4500–5000 *u*) and partially overlaps with the ionic signal of $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$. The masses of these species are consistent with unsaturated Hp/Hb complex (i.e., $Hp \cdot \alpha^{Ga}\beta^{Ga}$). Since the extent of multiple charging of these species is nearly the same as that of $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$, it seems highly unlikely that these ions represent products of gas-phase dissociation. Even though in some cases asymmetric charge partitioning is not the only channel of gas-phase dissociation of protein assemblies [47], transition from $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$ (charge states +27 through +30) to $Hp \cdot \alpha^{Ga}\beta^{Ga}$ (charge states +27 and +28) in the gas phase would require removal of hemoglobin dimer $\alpha^{Ga}\beta^{Ga}$ carrying very few charges from the multiply charged assembly, a process that appears to be thermodynamically unfavorable [46]. Although the solution-phase origin of the observed $Hp \cdot \alpha^{Ga}\beta^{Ga}$ species might seem to contradict the observation that the ionic signal intensity ratio $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2/Hp \cdot \alpha^{Ga}\beta^{Ga}$ decreases at higher collisional activation (see Supplementary Material for more detail), this is likely to be a consequence of $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$ ions being more prone to gas-phase dissociation (and suffering greater population loss when the collisional energy is increased).

While it might seem puzzling that the Hp/Hb^{Ga} complexes of different stoichiometries co-elute in SEC, one must remember that Hp has an extended, dumbbell-shaped conformation, and binding of hemoglobin does not result in a noticeable increase of its hydrodynamic radius [48]. A very modest increase of the extent of multiple charging in native ESI MS upon Hp association with either $\alpha^{Fe}\beta^{Fe}$ (as reported in [43]) or $\alpha^{Ga}\beta^{Ga}$ (as seen in Figure 4) is also consistent with a relatively insignificant increase of the physical size of the macromolecule [49]. Therefore, it should not be surprising that $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2$ and $Hp \cdot \alpha^{Ga}\beta^{Ga}$ cannot be separated by SEC. The practical ramifications of this (as related to the ultimate goal of our work) are quite significant, as the presence of the partially unsaturated Hp/Hb complex in the Ga-labeled sample means that the Ga/Hp molar ratio is less than the presumed ratio of 4:1. The magnitude of the correction can be estimated from the ionic signal intensity ratio $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2/Hp \cdot \alpha^{Ga}\beta^{Ga}$ in the native ESI mass spectrum acquired under mild conditions in the ESI interface region (such as that shown in Figure 4). Even though the relative ion current intensity of a particular species cannot be simply equated to its fractional concentration in solution [50], the correlation could be reasonably close, especially at lower protein concentrations [51]. Based on these considerations, we evaluate the $Hp \cdot (\alpha^{Ga}\beta^{Ga})_2/Hp \cdot \alpha^{Ga}\beta^{Ga}$ concentration ratio in solution as 3.3 :1, which corresponds to the Ga/Hp labeling ratio of 3.5 :1.

ICP-MS Detection of $Hp{\cdot}Hb^{Ga}$ in Human Serum

Knowing the Ga/Hp labeling ratio provides an opportunity to determine the concentration of this protein (or protein/drug conjugates) in complex biological matrices by measuring the levels of Ga. Ga can be readily detected in a variety of

biological matrices using ICP MS, although both of its stable isotopes are known to have spectral interferences, such as $^{138}Ba^{2+}$ for $^{69}Ga^{+}$, and $^{40}Ar^{31}P^{+}$ and $^{35}Cl^{36}Ar^{+}$ for $^{71}Ga^{+}$ [52, 53]. Figure 5 shows a relevant region of the ICP mass spectrum of a blank (human serum that had not been doped with Ga in any form), where low-abundance ionic signal is observed at both *m*/*z* 69 and 71, even though the measurements were carried out in the kinetic energy discrimination (KED) mode to reduce polyatomic interferences. Spiking the serum sample with Hp/Hb^{Ga} (final concentration of Hp in the sample is 8.4 nM) resulted in a noticeable increase of the ionic signal at both *m*/*z* values (see the red-filled curve in Figure 5). The signal intensity increase exceeds an order of magnitude, suggesting that Ga can be successfully used as a tracer in pharmacokinetic studies of Hp-based therapeutics (the protein therapeutics serum concentration range that needs to be accessible to measurements is estimated to span from sub-μM to low-nM levels [54]). Carrying out the measurements of Ga concentration in a common "hopping mode" (where only the ionic currents of isotopes of interest are measured, such as ^{69}Ga, ^{71}Ga, and the internal standards), as opposed to the continuous quadrupole-scanning mode (shown in Figure 5) will result in further increase of the measurement sensitivity (see Supplementary Material for more detail). This will allow exogenous Hp to be detected not only in circulation at concentrations that are significantly below typical levels of the endogenous protein in human plasma [55], but also in various tissues despite unfavorable tissue/serum ratios.

The goal of the present work was to develop a method of detecting and measuring exogenous Hp in complex biological matrices (such as blood plasma), with the subsequent aim of extending this approach to include Hp-based antiviral therapies. One particularly attractive target currently pursued in our laboratory is Hp conjugated to analogs of adefovir [56]. The latter is a small-molecule medicine that was initially designed as an HIV treatment, but the clinical studies showed that absent targeted delivery to macrophages, therapeutically active doses of adefovir are unsafe for patients. As a result, its clinical applications are now limited to the low-dose format to treat a lower value target, hepatitis B virus [57]. Conjugation of adefovir to the Hp/Hb complex appears to be a reasonable strategy to achieve the desired therapeutic outcome with a lower drug dosage by virtue of targeted delivery. Quantitation of adefovir/Hp/Hb conjugates in biological matrices could be also carried out with high specificity and sensitivity using Ga as a tag by introducing a slight modification to the procedure described in the present work. In this case, Ga/adefovir ratio can be determined by measuring the Ga/P ratio in the conjugate sample by ICP MS (despite interferences from $^{14}N^{16}O^{1}H^{+}$ and $^{15}N^{16}O^{+}$ [58], ICP MS allows phosphorus to be monitored with a detection limit in the sub-ppb range [59]). Lastly, the sensitivity of detection and quantitation of exogenous Hp or Hp/drug conjugates based on metal tags may be further improved by exploring the utility of other metals beyond gallium, since protoporphyrin IX can be substituted with a variety of non-ferric metals [60].

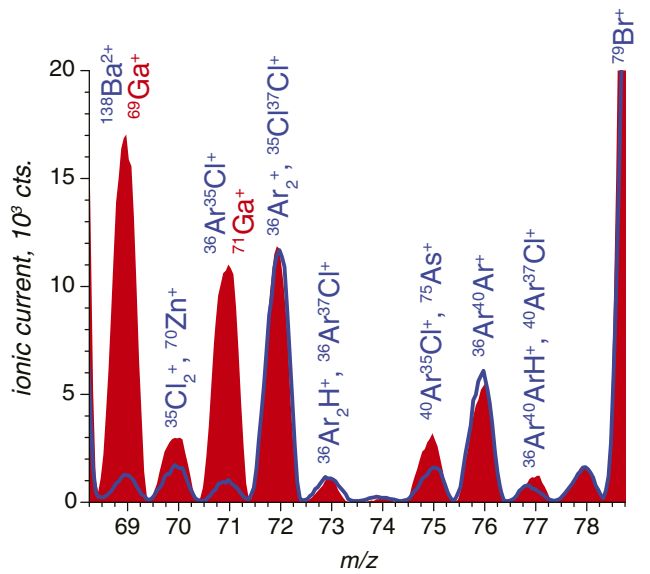

Figure 5. Zoomed views of full-scan ICP mass spectra of human serum before (blue trace) and after (red trace) addition of exogenous $Hp \cdot Hb^{Ga}$ complex (the final concentration of the protein in the sample is 8.4 nM)

Conclusions

Biopharmaceuticals remain one of the fastest growing sectors in modern medicine, but their continued success hinges upon the ability to provide detailed and reliable characterization of these complex therapeutic agents and their behavior in vivo. Collecting pharmacokinetics data is a particularly challenging task for several protein drug candidates, especially those that are structurally very similar or indeed identical to endogenous proteins, e.g., those that enable access to specific target sites. Hp is one of such proteins, as it can uniquely access macrophages, a trait that can be taken advantage of for the purpose of targeted drug delivery in a variety of antiviral strategies. Tracing exogenous Hp or Hp/Hb complexes in organisms can be very challenging, since these measurements have to be carried out on the background of abundant (an varying) endogenous Hp. We address this problem by substituting iron-bound heme group with the gallium-bound protoporphyrin IX in Hb prior to complexing it with Hp. While Ga-labeled free Hb appears to be only marginally stable, the Hp/Hb^{Ga} complex displays remarkable stability on a time scale relevant for pharmacokinetic measurements. Gallium is a non-essential metal and is not present in humans (absent any environmental or workplace exposure to Ga-containing materials), which makes it a convenient tag that is suitable for detection and quantitation of exogenous Hp/Hb^{Ga} complexes in biological matrices using ICP MS as a method of detection. Despite several spectral interferences, Ga can be detected with sensitivity that is more than adequate for pharmacokinetic studies of Hp-based therapeutics.

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