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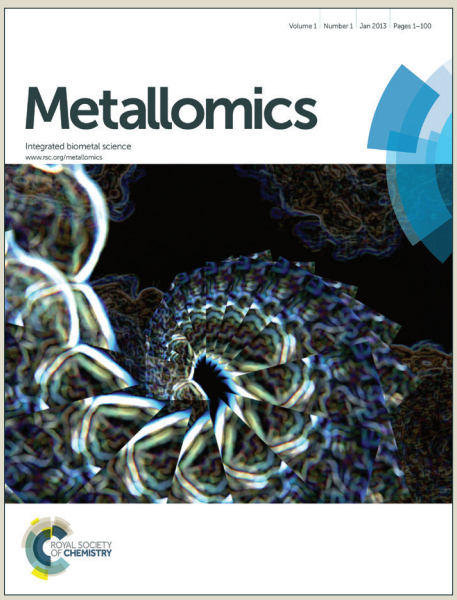

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A potential reference method for accurate and precise determination of ferritin bound iron as a new biomarker for body iron status.

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ARTICLE

Quantification of Ferritin Bound Iron in Human Serum by Species-Specific Isotope Dilution Mass Spectrometry

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Ferritin is a hollow sphere protein composed of 24 subunits that can store up to 4,500 iron atoms in its inner cavity. It is mainly found in liver and spleen but also in serum at trace levels. Serum ferritin is considered as the best single indicator in assessing body iron stores except liver or bone marrow biopsy. However, it is confounded by other disease conditions. Ferritin bound iron (FBI) and ferritin saturation have been suggested as more robust biomarkers. Current techniques for FBI determination are limited by low antibody specificity, low instrument sensitivity and possible analyte losses during sample preparation. The need for a highly sensitive and reliable method is widely recognized. Here we describe a novel technique to detect serum FBI using species-specific isotope dilution mass spectrometry (SS-IDMS). [^{57}Fe]-ferritin was produced by biosynthesis and *in vitro* labeling with ^{57}Fe spike in the form of [^{57}Fe]-citrate after cell lysis and heat treatment. [^{57}Fe]-ferritin for sample spiking was further purified by fast liquid protein chromatography. Serum ferritin and added [^{57}Fe]-ferritin were separated from other iron species by ultrafiltration followed by isotopic analysis of FBI using negative thermal ionization mass spectrometry. Repeatability of our assay is 8 % with an absolute detection limit of 18 ng FBI in the sample. As compared to other speciation techniques, SS-IDMS offers maximum control over sample losses and species conversion during analysis. The described technique may therefore serve as a reference technique for clinical applications of FBI as a new biomarker for assessing body iron status.

Introduction

Ferritin is among the oldest biomolecules on our planet[1]. Virtually all living organisms rely on it as their central iron storage protein to build up iron reserves for times of need or to store away excess iron safely. Unshielded ferrous iron can catalyze formation of hydroxyl radicals by Fenton/Haber-Weiss chemistry[2]. In humans, high iron status is discussed to play a role in the development of cardiovascular disease, diabetes type 2 and neurodegenerative disorders[3-5].

The ferritin molecule is a hollow sphere that can store up to 4,500 iron atoms in its inner cavity in the form of ferric-oxyhydroxy-phosphate. The sphere is made up from 24 H- and L-subunits with molecular weights of 21 kDa and 19 kDa, respectively, with the overall molecular weight ranging from 450 - 702 kDa depending on iron filling grade of the shell[6,7]. The H-subunit is associated with iron oxidation for uptake of iron into its cavity while the L-subunit is supposedly responsible for iron nucleation and storage[8]. Ferritin is mainly found in liver and spleen in humans. It is also present in serum and other tissues but at much lower concentration. Unlike tissue ferritin, serum ferritin comprises mainly of L-subunits with little H-subunits and its iron saturation is rather low[9-13].

In absence of inflammation and/or chronic disease, serum ferritin concentration is considered the best single indicator in assessing iron status except liver or bone marrow biopsy due to its less invasive assessment and specificity. Normal ranges set by WHO are 15 - 150 µg/L for adult women and 15 - 200 µg/L for adult males[14]. While serum ferritin concentration below the normal range is highly specific for diagnosis of iron deficiency, high serum ferritin concentration is not necessarily indicative of iron overload. As an acute phase protein, its serum concentration increases sharply in inflammatory conditions[15]. Here, serum concentration of soluble transferrin receptor (sTfR), its ratio relative to logarithmized serum ferritin concentration or a combination of both is of higher diagnostic value. Soluble transferrin receptor is not affected by non-iron related factors and is able to distinguish iron-deficiency anemia from anemia of chronic disease[16].

Ferritin bound iron (FBI) in serum has been suggested as another more robust marker for evaluation of iron status as it is supposedly less confounded by inflammation as compared to measurements of ferritin protein concentration[17]. This is supported by studies showing that iron saturation of serum ferritin is significantly lower in patients with acute phase response than in healthy humans[18,19]. Immunochemical techniques are commonly used to measure ferritin concentration in serum. However, such assays are only sensitive to the protein but not the iron bound inside the shell, which is highly variable due to differences in filling grade.

Initial attempts to measure FBI employed immunochemical separation of ferritin and subsequent measurement of FBI by graphite furnace atomic absorption spectrometry[17], coulometry[20] or colorimetric techniques[21]. However, the antibody required for ferritin separation is usually raised against liver or spleen ferritin, which has low specificity for serum ferritin. This affects recovery of serum ferritin and may result in analytical bias. Moreover, concentrations of FBI in serum are in the µg/L range at which accurate iron quantification is challenging. Konz et al. suggested recently, an alternative strategy using inductively coupled plasma mass spectrometry (ICP-MS)[10]. Simultaneous measurement of FBI and ferritin protein concentration were measured using a sandwich assay where two types of antibodies, one biotinylated and the other labeled with a stable ruthenium isotope (^{99}Ru) were used to separate and quantify ferritin in parallel to iron using isotope dilution techniques for post column quantification of the ruthenium tag. However, accurate quantification of ferritin and its iron content in this assay depends largely on the accurate determination of the ruthenium/ferritin stoichiometry. Moreover, sample losses before ^{99}Ru spiking after column chromatography may also affect the accuracy of the measured ferritin concentration and its iron saturation.

It is commonly agreed that species-specific isotope dilution mass spectrometry (SS-IDMS) is the method of choice for element speciation analysis[22], including FBI quantification. Once the isotopically labeled element species has equilibrated with the native element species in the sample, analyte losses or species conversion have no effect on measurement accuracy. Both are major sources of bias in available assays. In a proof-of-concept study we have demonstrated that ferritin protein can be over-expressed and filled with ^{57}Fe enriched iron *in vivo* in bacterial culture[23] and that the produced isotopic spike can be used for quantification of FBI by SS-IDMS in plant samples[23,24]. Here we have expanded the concept to the measurement of FBI in human serum samples with its substantially lower iron content and also introduce *in vitro* isotopic labeling of ferritin with ^{57}Fe for SS-IDMS as an alternative to our earlier *in vivo* approach.

Experimental section

Materials

Reagents were of analytical grade and high-purity (18 MΩ) deionized water (Millipore Elix®, Merck-Millipore, Billerica, MA, USA) was used in all experiments. Acids (65% HNO_3 and 37% HCl, Merck, Darmstadt, Germany) were purified further in house by sub-boiling distillation in quartz stills (SAP-900 IR, AHF Analysentechnik, Tübingen, Germany). Labware was acid washed (polyethylene and polypropylene, single use items) or boiled (Teflon PFA; multiple use items) in 10% (v/v) HNO_3. Isotopically enriched iron (95.63 ± 0.02 % ^{57}Fe) was purchased in metal form from Chemgas (Boulogne, France). Nitrilotriacetic acid (NTA) (BioUltra), Na_2CO_3 (ACS), iron exchange resin for iron separation (Dowex AG 1X8) and horse spleen ferritin (F4503) were obtained from Sigma-Aldrich (St. Louis, MO, USA). An iron isotopic reference material certified for iron isotopic composition (IRMM-014, EU Institute of Reference Materials and Measurements, Belgium) was used as a control material for iron isotopic analysis. Primary iron standard for assay calibration (Titrisol®) was obtained from Merck (Darmstadt, Germany).

Biosynthesis of apo-ferritin

Recombinant *Phaseolus vulgaris* ferritin was produced according to Hoppler et al with minor modification[23]. Briefly, *E.coli*/pPVF transformants of strain BL21-CodonPlus(DE3)-RIPL were cultured at 37°C in modified M9 media without any iron addition but kanamycin sulfate and chloramphenicol supplementation. After optical density of the cell suspension at 600 nm wavelength reached 0.6, isopropyl-β-thiogalactopyranoside (IPTG) was added to induce apo-ferritin synthesis. Cells were harvested 24 h after induction by centrifugation. Cells were disrupted by enzymolysis (B-PER II Bacterial Protein Extraction Reagent (2X), Thermo Scientific, Pittsburgh, PA, USA) and ultra-sonication (Fisher Scientific ™ Model 120 Sonic Dismembrator, Thermo Fisher Scientific, Waltham, MA, USA). In a second step, heat instable proteins were removed from the supernatant by warming to 63 °C for 13 min for precipitation. Subsequent centrifugation at 10,000 g for 45 min for removal of cell debris and denatured proteins yields ca. 10 mL heat treated lysate per 50 mL of cell culture.

Isotopic labeling of apo-ferritin

For ferritin labelling, a $^{57}FeCl_2$ solution (24 mg Fe/g) was prepared by dissolution of ^{57}Fe metal in 5 M HCl. Citric acid was added to prevent iron precipitation when adjusting the pH of the solution to 7.4 with 1 M Na_2CO_3. For 1 L of cell culture (= 200 mL heat treated lysate), 7.37 mL 1 M citric acid solution was added to a 0.42 g aliquot of the $^{57}FeCl_2$ solution to give a final molar ratio of iron to citrate of 1:4. The prepared [^{57}Fe]-citrate solution was added to ca. 10 mL of the heat treated cell lysate. Excess [^{57}Fe]-citrate was removed by ultra-filtration (Amicon, 30 kDa NMWL; Merck-Millipore, Billerica, MA, USA) at 4,500 g after 1 h incubation at room temperature. The filter was rinsed with 10% HNO_3 and water three times prior to use.

Optimal conditions for isotopic labeling were tested out before using citric acid and NTA as possible iron chelators. Solutions of $FeCl_2$ of natural isotopic composition (24 mg Fe/g) were mixed with 1 M aqueous solutions of citric acid and NTA, respectively, to obtain a final molar ratio of iron to chelator of 1:4. The pH of the solutions was adjusted to 7.4 with 1 M Na_2CO_3. The heat treated *E.coli* lysate containing recombinant apo-ferritin was prepared as described before and different amounts of iron (100, 500, 1000, 2500 µg) were added to 10 mL aliquots of the lysate. Aliquots were incubated at room temperature for 1 h and 24 h, respectively. Non-FBI was removed by ultra-filtration (Amicon Ultra 30 kDa). Iron amount in the retentate was determined from the mass of the retentate and iron concentration as determined by graphite furnace atomic absorption spectrometry (GF-AAS; Zeeman Atomic Absorption Spectrometer AA240Z, Agilent Technologies, Santa Clara, CA, USA) after microwave digestion using 8 mL HNO_3 and 2 mL H_2O_2 in a microwave oven (ETHOS One; Milestone, Sorisole, Italy). Iron uptake efficiency was calculated as the ratio of iron in the retentate to

total iron added to the bacterial culture. A subsample of the retentate was used to confirm the presence of iron in recombinant ferritin by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and native PAGE.

Purification of [^{57}Fe]-ferritin

Samples were applied for purification to a diethylaminoethyl (DEAE) Sepharose column (10 cm × 1.6 cm; GE Healthcare, Uppsala, Sweden) followed by size exclusion chromatography using a Sephacryl S-300 column (2.6 cm × 60 cm; GE Healthcare, Uppsala, Sweden). Ferritin containing fractions were combined and concentrated by ultra-filtration and stored at 4°C. Production and purity of recombinant ferritin after separation were confirmed by SDS-PAGE and native PAGE. Iron in ferritin was stained with Prussian blue (2 % $K_4Fe(CN)_6$ solution in 2 % HCl) and ferritin was stained with Coomassie blue using standard protocols.

Table 1 Operating Conditions for ferritin purification by fast protein liquid chromatography (FPLC)

Anion exchange column	DEAE Sepharose (10 cm × 1.6 cm)
Mobile phase	(A) 25 mM Tris-Cl buffer (pH 7.4) (B) A + 0.5 M NaCl
Gradient	0-100 % B within 5 column volumes
Flow rate	4 mL/min
Size exclusion column	Sepharcryl S-300 column (2.6 cm × 60 cm)
Mobile phase	50 mM phosphate buffer + 0.5 M NaCl (pH 7.4)
Flow rate	1 mL/min

Characterization of [^{57}Fe]-ferritin

The purified [^{57}Fe]-ferritin solution was characterized for iron isotopic composition using negative thermal ionization mass spectrometry (NTI-MS) after microwave digestion and iron separation by ion exchange chromatography (see below). Concentration of the ^{57}Fe label was determined by reversed IDMS using similar procedures after spiking of [^{57}Fe]-ferritin aliquots with a commercial iron standard of known iron concentration (Titrisol®; Merck, Darmstadt, Germany).

Assessment of species conversion

An aliquot of the purified [^{57}Fe]-ferritin solution containing 5 µg ^{57}Fe was mixed with 5 µg of natural iron (IRMM-014) as Fe-citrate at pH 7.4 and incubated for 60 min at room temperature. The mixture was subjected to ultrafiltration (Vivaspin, 300 kDa; Satorius AG, Göttingen, Germany). The Vivaspin filter was rinsed with 10% HNO_3 and high-purity (18 MΩ) deionized water three times prior to use. The cut-off value of the filter was chosen to be well below the molecular weight of ferritin (> 450 kDa) and much higher than the molecular weight of the second largest iron species in serum which is transferrin (80 kDa). Iron isotope ratios in the retentate ([^{57}Fe]-ferritin fraction incubated with natural iron) were analyzed using NTI-MS (see below) and compared with the isotope ratios in the [^{57}Fe]-ferritin solution not incubated with natural iron. Similar behavior of native ferritin and [^{57}Fe]-ferritin was verified by spiking a serum sample with [^{57}Fe]-ferritin and separation of ferritin and non-ferritin fractions by ultrafiltration using a Vivaspin filter. The isolated ferritin was subjected to ultrafiltration for a second time in an identical manner. The iron isotopic composition of the ferritin mixture before and after the second separation was compared to confirm identical behavior of labeled and native ferritin during species separation.

Analysis of FBI in serum

Serum was obtained from an apparently healthy, male adult volunteer of high iron status (hemoglobin: 175 g/L, serum ferritin: 443 µg/L; transferrin: 2.79 g/L; transferrin saturation: 49%; total iron binding capacity: 4.08 mg/L; serum iron: 2.00 mg/L) not suffering from an inflammatory condition (C-reactive protein: < 5 mg/L). The subject was approached and informed about aims and procedure of the study as well as possible risks of study participation orally and in writing and written informed consent was obtained. Iron status parameters were assessed using automated clinical analyzer techniques at the National University Hospital, Singapore. Whole blood (ca. 75 mL) was drawn into trace metal free vacutainers (Vacutainer® Trace Element; Becton and Dickinson Company, Franklin Lakes, NJ, USA) after an overnight fast and serum was separated by centrifugation at 3,000 rpm for 15 min after clotting. Serum samples were pooled, mixed well and divided into 3 aliquots of 12 mL each for triplicate analysis. Each serum aliquot of serum sample was spiked with recombinant [^{57}Fe]-ferritin containing 5 µg Fe and serum ferritin was isolated from other iron-containing species by ultrafiltration (Vivaspin, 300 kDa; Satorius AG, Göttingen, Germany) at 4,500 rpm for 20 min six times to ensure the complete removal of other iron species. The filter was rinsed with 10% HNO_3 and high-purity (18 MΩ) deionized water three times prior to use. Purification efficiency was tested using SDS-PAGE. Iron isotopic composition of isolated ferritin was measured after sample digestion and iron purification as detailed below. All experiments were performed in compliance with relevant laws and university guidelines. The protocol was reviewed and approved by the NUS Institutional Review Board for human studies.

Iron isotope analysis by NTI-MS

Ferritin fractions collected after ultrafiltration were mineralized using 8 mL HNO_3 and 2 mL H_2O_2 in a microwave oven. Iron was separated from the sample matrix by anion exchange chromatography (Dowex AG-1X8) and further purified by ammonium hydroxide precipitation as described elsewhere[25]. Iron isotopic composition was determined by NTI-MS using FeF_4^- molecular ions and a rhenium double-filament ion source[26]. Briefly, both the evaporation as well as the ionization filament was coated first with BaF_2 to promote the formation of negatively charged ions. Sample iron in 40 % (w/w) HF was then loaded as FeF_3 on top of the BaF_2 layer on the evaporation filament and coated with a solution of $AgNO_3$ in HF (20 %). All mass-spectrometric measurements were carried out with a magnetic sector field mass spectrometer (Triton; Thermo-Finnigan, Bremen, Germany) equipped with a multi-collector system comprising of nine Faraday Cups and automated amplifier rotation to correct for differences in amplifier gain. Analytical blanks were measured by processing known amounts of pure ^{57}Fe spike for the entire analytical run including sampling and ultra-filtration.

Calculation of ferritin iron saturation

FBI concentration (µg/L) was divided by ferritin concentration (µg/L) and converted to iron saturation of intact ferritin (%) using a conversion factor of 179.2 according to

Kate et al. assuming a maximum capacity of 4,500 Fe atoms per ferritin molecule[18]. Average number of Fe atoms per ferritin molecule was obtained by dividing FBI concentration (µmol/L) by ferritin concentration (µmol/L) in the sample.

Results and discussion

Results

OPTIMIZATION OF IRON LOADING OF RECOMBINANT APO-FERRITIN. The amount of iron taken up by recombinant apo-ferritin increased as the added iron amount was varied upwards with a more pronounced increase up to 1,000 µg added iron (see Fig. 1a). Iron uptake efficiency decreased in near exponential fashion with increasing iron load in the culture. Fe-citrate was found to be a better iron donor at all added iron amounts compared to Fe-NTA (see Fig. 1b) which can be attributed to its lower affinity to iron as compared to NTA[27]. No significant differences were found between incubation times of 1 h and 24 h (data not shown). Considering both absolute iron uptake and uptake efficiency, an iron load of 500 µg iron as Fe-citrate per 50 mL culture and an incubation time of 1 h were found to be optimal for ferritin saturation. Native PAGE with Prussian blue staining confirmed that the iron found in the solution was in the form of FBI and not bound non-specifically to other proteins (see Fig. 2). The bands appeared above the main ferritin band in *lane 1* of Fig.2 which was probably due to the formation of dimers, trimers or oligomers in the horse spleen ferritin standard which is a common phenomenon associated with ferritin ageing[28].

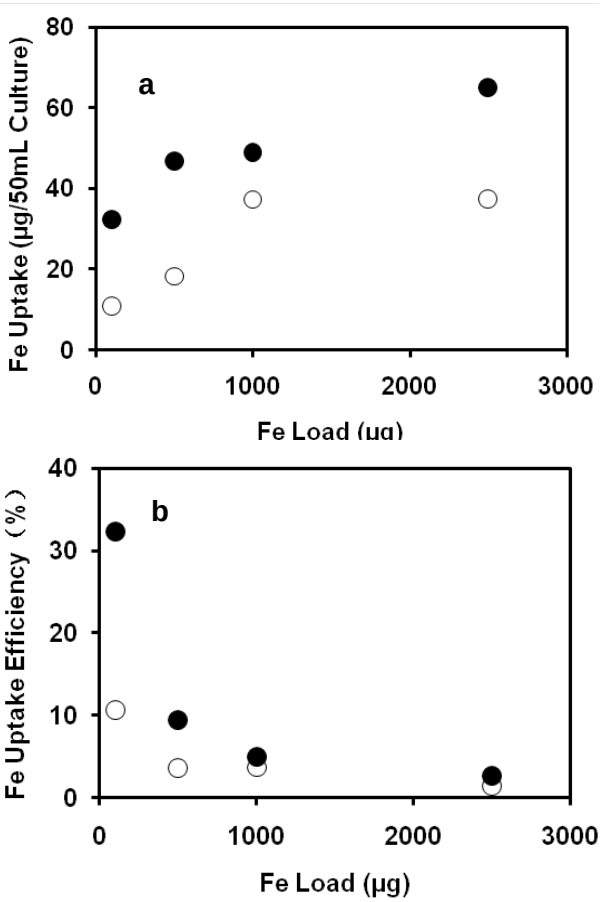

Fig. 1 Effect of iron load on *in vitro* uptake of iron by recombinant ferritin from *E.coli*/pPVF: (a) absolute amount of iron taken up by apo-ferritin (per 50 mL culture) from Fe-citrate (●) and Fe-NTA (○); (b) fraction of added iron taken up by apo-ferritin from Fe-citrate and Fe-NTA, respectively. Error bars (1 SD) are smaller than plotted symbol sizes.

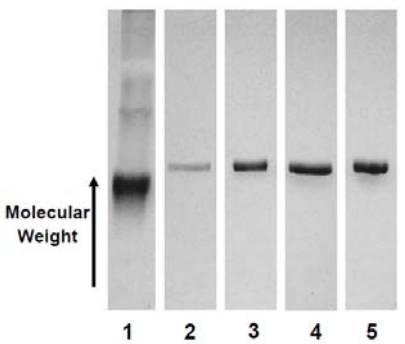

Fig. 2 Native PAGE (5% polyacrylamide gel) of recombinant apo-ferritin from *E.coli*/pPVF after iron loading with Fe-citrate *in vitro* at different iron concentrations and iron staining using Prussian blue. *Lane 1:* horse spleen ferritin (control); *lane 2:* 100 µg added iron per 50 mL culture; *lane 3:* 500 µg added iron per 50 mL culture; *lane 4:* 1,000 µg added iron per 50 mL culture; *lane 5*: 2,500 µg added iron per 50 mL culture.

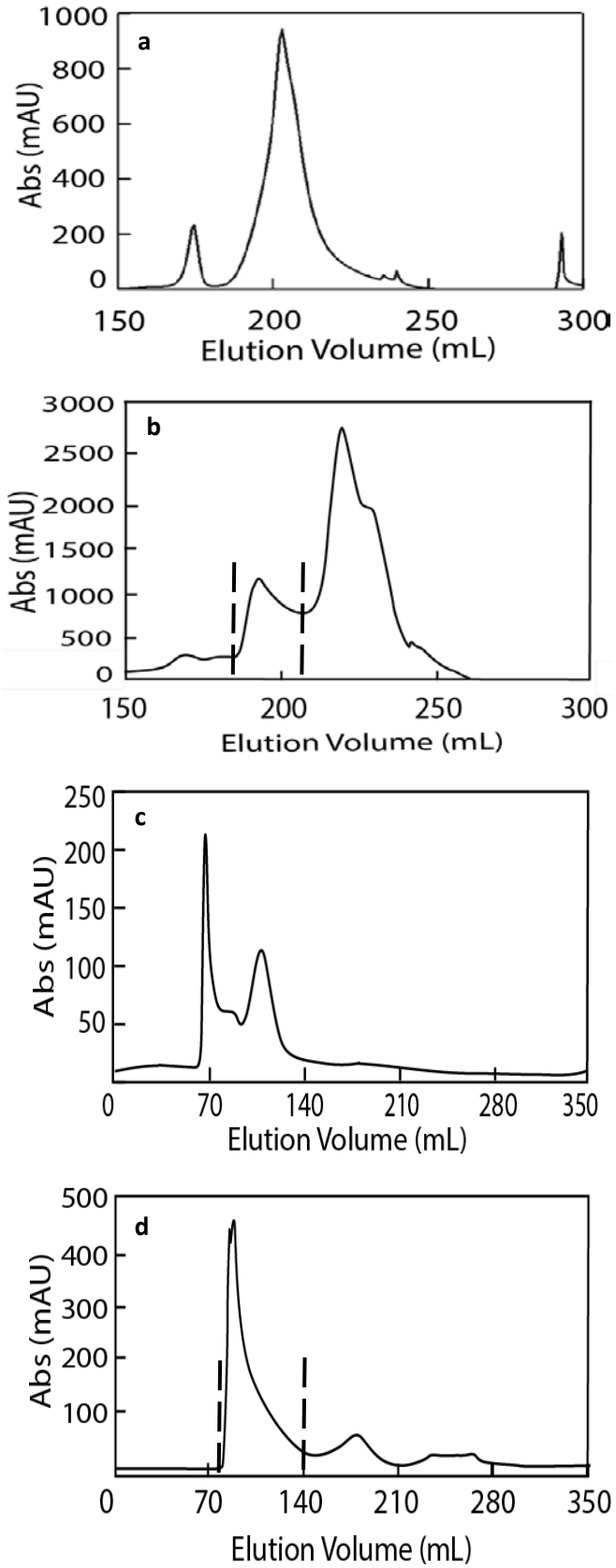

Fig. 3 Anion exchange chromatograms of (a) commercial horse spleen ferritin and (b) *E.coli* lysate after heat treatment. Size exclusion chromatograms of horse spleen ferritin and *E.coli* lysate after anion exchange are shown in Figs. 3c and 3d, respectively. Fractions of the lysate in Figs. 3b and 3d containing [^{57}Fe]-ferritin were identified by retention time for further characterization by PAGE. Analysed fractions are indicted by dashed line. Multiple peaks in the horse spleen ferritin chromatograms are probably due to presence of ferritin oligomers and impurities. Differences in retention times between horse spleen ferritin and recombinant plant ferritin can be attributed to differences in size and isoeectric points..

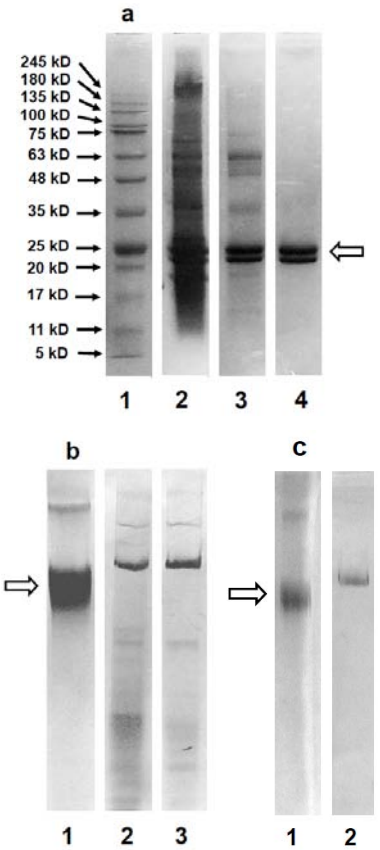

Fig. 4 Expression, purification and iron content of recombinant ferritin from *E.coli*/pPVF. **a** SDS-PAGE (12% polyacrylamide gel) of protein extract after Coomassie blue staining. *Lane 1*: Markers; *lane 2*: *E.coli* lysate; *lane 3*: *E.coli* lysate after anion exchange chromatography; *lane 4*: *E.coli* lysate after size exclusion chromatography. **b** Native PAGE (5% polyacrylamide gel) of protein extract after Coomassie blue staining. *Lane 1*: horse spleen ferritin; *lane 2*: *E.coli*/pPVF cell extract; *lane 3*: *E.coli*/pPVF cell extract after heat treatment. **c** Native PAGE (5% polyacrylamide gel) of protein extract after Prussian blue staining. *Lane 1*: horse spleen ferritin; *lane 2*: *E.coli*/pPVF cell extract after *in vitro* iron loading and the entire purification procedure. The two ferritin subunits in 4a and intact ferritin in 4b and 4c are marked with an *arrow*. Ferritin is preserved in native PAGE but disintegrates into its subunits in denaturing SDS-PAGE.

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF RECOMBINANT [^{57}Fe]-FERRITIN. The FPLC chromatograms of the horse spleen ferritin standard and *E.coli* cell lysate are shown in Fig. 3. Fractions of the lysate containing [^{57}Fe]-ferritin were identified by retention time and were subjected to further characterization by PAGE. Observation of an iron protein of high molecular weight in native PAGE that disintegrates into two different proteins in the 20-35 kDa range under the denaturing conditions of SDS-PAGE is a unique feature of ferritin. In good agreement, SDS-PAGE showed two proteins in the 20-25 kDa range (see Fig.4a, *lanes 2-4*) while native PAGE showed a compound of high molecular weight

that stained positive for protein (see Fig.4b, *lane 2*) as well as iron (see Fig.4c, *lane 2*). Heat treatment of the cell lysate was effective in removal of other heat unstable proteins without affecting ferritin (see Fig.4b, *lane 2* and *3*). Iron-containing proteins other than ferritin could successfully be removed by anion exchange and size exclusion chromatography as verified by SDS-PAGE (Fig.4a, *lane 4*) and native PAGE (Fig.4c, *lane 2*) of the cell lysate after chromatographic purification. Differences in band position between recombinant and horse spleen ferritin in PAGE (*Lane 1* & *2* of Figs. 4b and c) and retention times in Fig. 3 could potentially be attributed to differences in their molecular weights and isoelectric points, The isotope ratio of $^{56}Fe/^{57}Fe$ of the purified ferritin was 0.08098 ± 0.00027 (n = 5). With the ratio being distinctly different from its analyzed value for natural iron (43.31 ± 0.02) and very similar to its value in the isotopically enriched iron metal (0.08827 ± 0.00003) we can conclude that the isotopic labeling was successful. Iron concentration in the ferritin solution was 32.18 ± 0.23 mg/L (n = 5) as determined by reversed IDMS using NTI-MS.

Separation of serum ferritin by ultrafiltration. SDS-PAGE showed that transferrin as the main iron-containing species (Fig.5, *lane 4*) and other low molecular weight proteins could be successfully removed by ultrafiltration, leaving only high molecular weight proteins in the retentate (Fig.5, *Lane 3*). Dissociated ferritin sub-units are not visible in *Lane 3* in Fig.5 because concentrations were below the detection limit of Coomassie blue staining.

Assessment of species conversion. The measured $^{57}Fe/^{56}Fe$ isotope ratios were 12.35 ± 0.04 (n = 5) and 12.31 ± 0.04 (n = 5) before and after incubation of isotopically labeled [^{57}Fe]-ferritin with natural iron for 60 min. This showed that iron species conversion, a major source of bias in SS-IDMS, was negligible. The measured $^{57}Fe/^{56}Fe$ isotope ratio of ferritin isolated from human serum and spiked with [^{57}Fe]-ferritin was 3.67 ± 0.45 and 3.66 ± 0.41 (n = 3) before and after ultrafiltration. This demonstrates absence of iron exchange between [^{57}Fe]-ferritin and native serum ferritin and that both species behave identically during separation.

Determination of FBI in human serum by SS-IDMS. For final assessment of the developed method, serum samples from a volunteer with high iron stores were analyzed. Serum FBI was 77.3 ± 6.4 µg/L (n = 3). At a serum ferritin concentration of 443 µg/L, this translates to an average iron load of 1,406 ± 116 Fe atoms per ferritin molecule and a saturation of the ferritin molecule of 31.3 ± 2.6 % of its maximum iron uptake capacity of 4,500 atoms (n=3). Coefficient of variation (CV) was 8 % for triplicate analysis. The absolute detection limit for the assay was found to be 18 ng for FBI based on a total sample processing blank of 12 ± 6 ng (n = 5) as determined by processing a known amount of pure ^{57}Fe spike for the entire analytical run including sampling and ultrafiltration.

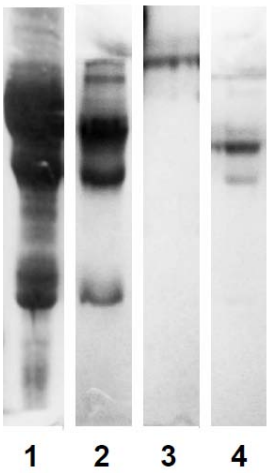

Fig.5 Evaluation of ferritin separation from serum by ultrafiltration using SDS-PAGE (12% polyacrylamide gel) and Commassie blue staining. *Lane 1*: serum; *lane 2*: filtrate; *lane 3*: retentate; *lane 4*: apo-transferrin.

Discussion

The amount of iron that is bound to ferritin in serum has significant potential as a biomarker of iron status as it is supposedly less sensitive to inflammatory conditions than the ferritin protein itself. This potential, however, remains largely unexplored due to the analytical difficulties inherent to its measurement. Concentrations of FBI in serum are in the low µg/L range which makes its analysis naturally a challenge in terms of instrument sensitivity and contamination control [9-13,17-21]. At an average concentration of serum iron of 1 mg/L, whole blood iron of 500 mg/L[29] and a typical FBI concentration of 20 µg/L[21], FBI constitutes less than 0.004 % of iron in whole blood and 2 % of iron in serum. This makes FBI separation from other iron species and species conversion, i.e. uptake or release of iron from ferritin during the analytical process, a major source of analytical bias.

SS-IDMS is widely regarded as the gold standard for elemental speciation. For elemental analysis using IDMS, analyte losses during storage and sample preparation do not affect the accuracy of the result[22,30]. Once the isotopically enriched spike and the native element in the sample have been completely mixed, any aliquot taken from the isotope-diluted sample contains spike and natural element in the same proportion. Similar to the use of an internal standard, the amount ratio of spike to natural element and, thus, the measurable isotope ratios in the sample carry the analytical information.

In SS-IDMS, not the element but the element species of interest is added in isotopically labeled form as a spike. Once sample and spike have equilibrated, neither sample losses during sample preparation or species conversion affect the accuracy of the result provided that both the native and the added isotopically labeled species are affected to the same extent[22,30]. The challenge of using SS-IDMS for metallo-protein analysis, however, is the need to incorporate the stable isotope into the metallo-protein either *in vivo* or *in vitro*. In our study

we used recombinant plant apo-ferritin obtained from genetically modified *E.coli* from an earlier study for spike preparation[23]. Because iron species are separated in our assay only by size, any isotopically labelled ferritin could have been used for SS-IDMS analysis provided that no species conversion occurs during analysis and labelled and unlabelled ferritin behave identically during ultrafiltration, which we have demonstrated. This entails that the use of the developed SS-IDMS method is not limited to human serum samples but can also be adopted for FBI analysis of samples containing other forms of ferritin including tissues of animal or plant origin.

The apo-ferritin purified from the *E.coli* lysate was loaded with ^{57}Fe *in vitro* instead of *in vivo* labeling during fermentation since the *in vitro* loading was found to deliver recombinant ferritin with higher iron saturation of up to 30 % (see Fig.1). The potential mechanism of iron loading of ferritin has already been elucidated. Ferrous iron binds first to the catalytic sites of apo-ferritin, which are supposed to be located either at the surface, along the channels penetrating the protein sphere or its inner cavity. Upon binding it is oxidized to ferric iron by the H-subunit in the presence of oxygen. The L-subunit is supposed to promote nucleation and polymerization[31]. While FBI analysis by SS-IDMS does not require a [^{57}Fe]-ferritin spike of high purity, it must be the only chemical form of the ^{57}Fe label in the spike solution in order to follow the native sample ferritin closely during the entire analytical process. Native PAGE analysis with iron staining revealed that the prepared spike solution contains no detectable amounts of other iron species than recombinant [^{57}Fe]-ferritin (see Fig.4c, *lane 2*). As in earlier studies, recombinant plant ferritin was found to migrate faster than horse ferritin in native PAGE[24]. Nevertheless, the band observed in *lane 2*, Fig.4c, could be safely identified as ferritin as there is no other iron species in higher organisms of such high molecular weight that disintegrates into two different subunits in the 20 kDa mass range under denaturing conditions (Fig.4a, *lane 3 & 4*). The weak band observed above the plant ferritin band can be assumed to be its dimer or trimer as reported previously[24].

For evaluation of measurement accuracy, neither a reference material certified for FBI concentration nor a highly purified ferritin standard of defined iron content for spiking experiments are currently available. To prepare and characterize such a standard using the techniques presented in this paper would have resulted in circular validation. Accordingly, we assessed measurement accuracy based on first principles by studying species conversion as the dominant source of Type B uncertainty in SS-IDMS according to GUM[32]. IDMS itself is considered a definitive technique, i.e. all sources of uncertainty in the analytical process other than species conversion can be quantified and their contribution to the measurement result assessed[33]. No measurable species conversion during sample preparation was observed in the conducted experiments. Furthermore, we could demonstrate that the isotopically labeled ferritin and native ferritin in serum follow each other closely during species separation by ultrafiltration. Ultrafiltration was preferred to chromatographic techniques due to its technical simplicity and the fact that no other known iron species than ferritin exists in human serum with a molecular weight higher than the cut-off of the used filter (300 kDa).

Combined measurement uncertainty was assessed using Monte-Carlo Simulations[34]. Isotopic composition of the sample, isotopic composition and iron concentration of the [^{57}Fe] ferritin spike, the measured ^{57}Fe/^{56}Fe iron isotope ratio of the blend and the processing blank were used as input variables together with their measurement uncertainties. Simulations were conducted for spiking ratios up to 100:1 for spike to natural iron in the blend. Estimated combined relative measurement uncertainties (k = 1) were of the order of 7-8 % and were minimally affected by the ratio of spike to sample in the blend up to a mixing ratio of 100:1. This estimate compares well with the assay repeatability of ca. 8% (1 RSD) we have measured. This level of analytical uncertainty can be considered acceptable for an iron species that is supposedly subject to strong inter-individual variations, which was evidenced in the study by Herbert et al[17] who reported variations of up to 250% in FBI concentration between individuals.

Variability in the processing blank was found to be the dominant contributor to measurement uncertainty which is not an unexpected observation. The measured processing blank was 12 ± 6 ng FBI at an absolute amount of 77.3 ± 6.4 ng FBI per analyzed serum aliquot, i.e. 8-23% of the FBI value as determined by SS-IDMS. As the processing blank can be hardly lowered further, possibilities for improving the assay are naturally limited. ICP-MS offers a higher mass spectrometric sensitivity for iron which would allow working with smaller serum samples and making over-spiking of samples as practiced in this work redundant. However, blank contributions would increase proportionally in this approach at the moment that the amount of iron coming from the sample is lowered. With the sample processing blank being the limiting factor, mass spectrometric precision is less decisive in terms of analytical quality. This means that more widely available single collector instruments, namely for ICP-MS, become a convenient and less costly alternative than MC-TIMS or MC-ICP-MS to open up this assay to a wider scientific community and for using it in clinical applications.

Serum FBI concentration in a healthy male volunteer with high iron stores was analyzed using our novel technique as a proof of concept. It was found to be 77.3 ± 6.4 µg/L (n = 3) which translates to an average iron load of 1,406 ± 116 Fe atoms per ferritin molecule and a saturation of the ferritin molecule of 31.3 ± 2.6 %. This is in good agreement with the study by Pootrakul et al.[35] who observed serum FBI concentrations below 100 µg/L when serum ferritin was below 750 µg/L. In another study, Herbert et al.[17] suggested that values between 35 µg/L and 100 µg/L are indicative of a positive iron balance which can be assumed for the studied volunteer based on the assessment of his iron status using established biomarkers. Lower FBI concentrations ranging from 11.2 µg/L to 22.4 µg/L were reported by Kate et al.[19] for healthy volunteers having serum ferritin concentrations

between 102 μg/L to 170 μg/L. Ferritin saturation in another study by the same author was found to be 24% at an average serum ferritin concentration of 112 μg/L[17]. In a recent study by Konz et al. (2013), measured FBI concentrations were below 30 μg/L at serum ferritin concentrations of volunteers up to 800 μg/L[10]. However, volunteers having serum ferritin concentrations higher than 200 μg/L were very few as in previous studies which make it difficult to compare these data with our results. Possible discrepancies can be explained furthermore by differences in gender and population groups between studies. However, they could also be due to inaccuracies in serum ferritin analysis and incomplete ferritin recovery during purification. Currently employed techniques for FBI analysis are all based on the use of immunochemical techniques where antibody specificity is highly problematic. Antibodies used for ferritin isolation are usually raised against liver or spleen ferritin, resulting in low specificity for serum ferritin due to the variation in the primary amino acid sequence and differences in the ratio of H- and L-subunit between ferritins of different physiological origin[36]. This low specificity can potentially affect serum ferritin recovery during isolation from other proteins thereby resulting in underestimation of its iron content at the moment that immunochemical techniques are employed. This disadvantage also limits the usefulness of ferritin saturation as a reliable iron status indicator as serum ferritin analysis is affected by this very same restriction.

Methods for iron quantification after ferritin purification in conventional assays include atomic absorption spectrometry (AAS)[17], coulometric techniques[20] and colorimetric methods[21]. The low concentration of serum ferritin and its low iron content makes iron quantification by AAS and colorimetric methods difficult because of their limited sensitivity in addition to matrix effects. The novel technique developed by Konz et al.[10] addresses some of these problems but it requires the accurate determination of the ruthenium/ferritin stoichiometry. Moreover, sample losses before ^{99}Ru spiking may also affect the accuracy of the ferritin concentration and ferritin saturation measurement. Post-column IDMS as used by Konz et al.[10] involves addition of the iron isotopic spike after ferritin isolation which may underestimate FBI content of the sample due to incomplete serum ferritin recovery. This is not the case for the presented SS-IDMS assay where [^{57}Fe]-ferritin is spiked into the sample directly after sampling. In the present work we could not observe any measurable discrimination between the [^{57}Fe]-ferritin and serum ferritin during isolation which means that sample losses will not affect measurement accuracy since any loss affects the [^{57}Fe]-ferritin spike and sample ferritin to the same extent. In addition, the SS-IDMS assay does not involve the use of any antibody, thereby avoiding the long continuing problem of low specificity of immunochemical based assays. As such, the presented method for FBI analysis can serve as a reference method for validation of less sophisticated assays for clinical applications. A major drawback of the presented assay is the rather large volume of blood required for analysis (ca. 20 mL whole blood) and the rather tedious sample preparation procedure including recombinant ferritin generation, *in vitro* iron loading and purification of the [^{57}Fe]-ferritin spike, species separation, sample digestion, iron purification and isotopic analysis.

The robustness of SS-IDMS in terms of species conversion and analyte losses during sample analysis makes SS-IDMS the method of choice in element speciation analysis when analytical accuracy and precision is a major concern. This refers in particular to metallo-protein analysis which is naturally challenging due to low concentration levels in clinical samples and needs to separate the protein of interest from usually complex sample matrices. So far, only a very limited number of attempts have been made to employ SS-IDMS for quantification of metallo-proteins[24,37-44]. The developed technique for measuring FBI in serum may serve as an example to further illustrate the potential of SS-IDMS in biomedical research and the possibilities arising from its use to identify and study new biomarkers in health and disease.

Conclusions

We have developed and successfully applied a novel technique to determine FBI concentration in human serum using SS-IDMS. This novel technique may serve as a reference technique since it is independent of serum ferritin recovery during the entire analytical procedure. The developed technique permits accurate and precise assessment of FBI and can be used to estimate ferritin saturation in human serum in combination with conventional immunochemical techniques for ferritin protein quantification. Thus, the analytical foundations have been laid for further exploration of FBI as a potentially more robust marker for assessment of body iron status than serum ferritin concentration in individuals and populations suffering from infection or imflammatory conditions.

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Notes and references

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