A nano-disperse ferritin-core mimetic that efficiently corrects anemia without luminal iron redox activity

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Abstract

The 2-5 nm Fe(III) oxo-hydroxide core of ferritin is less ordered and readily bioavailable compared to its pure synthetic analogue, ferrihydrite. We report the facile synthesis of tartrate-modified, nano-disperse ferrihydrite of small primary particle size, but with enlarged or strained lattice structure (~2.7 Å for the main Bragg peak versus 2.6 Å for synthetic ferrihydrite). Analysis indicated that co-precipitation conditions can be achieved for tartrate inclusion into the developing ferrihydrite particles, retarding both growth and crystallization and favoring stabilization of the cross-linked polymeric structure. In murine models, gastrointestinal uptake was independent of luminal Fe(III) reduction to Fe(II) and, yet, absorption was equivalent to that of ferrous sulphate, efficiently correcting the induced anemia. This process may model dietary Fe(III) absorption and potentially provide a side effect-free form of cheap supplemental iron.

From the Clinical Editor: Small size tartrate-modified, nano-disperse ferrihydrite was used for efficient gastrointestinal delivery of soluble Fe(III) without the risk for free radical generation in murine models. This method may provide a potentially side effect-free form iron supplementation.

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Despite all attempts to eradicate or even reduce iron deficiency anemia (IDA), it remains the most prevalent nutritional deficiency disorder in the world affecting around 1 billion people, of which nearly 400 million are children.\textsuperscript{1} In childhood, IDA is associated with increased morbidity and reduced cognitive development and IDA remains on the World

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Unlike most nutritional deficiencies, IDA is not confined to underdeveloped and developing countries: in the UK, for example, IDA affects around 5% of adults, nearly 12% of pre-school children and, in some areas, up to 25% of school children.1,5,6

To help combat IDA, oral iron supplementation needs to be well tolerated, cheap, safe and effective but current preparations fail in at least one of these criteria. Simple ferrous iron [Fe(II)] salts are most commonly used as these are inexpensive and the iron is well absorbed. However, they have lately come under the spotlight in high-profile intervention trials as they may enhance systemic infection rates, induce undesirable changes to commensal bacteria of the colon and increase pro-inflammatory signaling of the gut epithelium.7-10 Some forms of ferric iron [Fe(III)] (e.g. ferric pyrophosphate) are considered safer and better tolerated in the gut lumen than Fe(II) but are poorly absorbed.11-14 Chelation may overcome the issue of oral Fe(III) bioavailability but then marked enhancement of colon cancer risk is seen in rodent models using chelates of Fe(III) EDTA15 or Fe(III) citrate16 for example. In pursuit of strategies for optimal oral Fe supplementation we considered the chemical speciation of dietary Fe(III) and its processing in the gastrointestinal tract. In this respect, carefully designed studies have shown that Fe(III) is well absorbed from ferritin.17,19 Ferritin is the broadly conserved biological storage form of iron and, thus, is found in both meat- and plant-based foods of the human diet: it is composed of a protein shell with a ferrihydrite-like mineral core.20,21 Ferrihydrite is a poly oxo-hydroxide Fe(III) nanoparticle (about 2- to 5-nm diameter) with a precise atomic structure that still remains a matter of debate because its size and crystallographically defective nature make its definitive characterization challenging.22-27 Notably, in ferritin, the surface of the ferrihydrite-like core appears destabilized (i.e. amorphous) compared to synthetic or geological ferrihydrite20 which must be related to interactions between the ferritin protein shell and the ferrihydrite surface and may involve a phosphate-rich surface layer.28 Indeed the morphology of the ferritin mineral core has been shown to follow the symmetry of the protein shell.20,29 Such surface destabilization probably ensures that iron in ferritin is abnormally labile for an oxo-hydroxide in the presence of protons or ligands: thus it is rapidly turned over in the cell lysosome and will solubilize in gastric acid17,30,32 However, it is a matter of debate as to whether the protein shell and ferrihydrite-like core of ferritin control the slow release of Fe(III) ions from the gastric environment, or, if ferritin as a whole or as a protein-denatured mineral nanoparticle is rapidly emptied from the stomach and endocytosed by the intestinal epithelial cell and broken down intralysosomally.18,32,33 Either way ferritin is an effective, bioavailable form of dietary iron that has, for example, led to considerable investment by the Global HarvestPlus Initiative in “bio-fortification” which involves the selection of staple food crops with enriched ferritin expression.34,37

Iron supplementation with pure ferritin would be immensely challenging due to the required scale of purification or the complexity of in vitro synthesis, and thus overall costs. However, synthetically, it may not be necessary to exactly mimic the ferritin molecule. When organic molecules are present during the pH-induced formation of averted ferrihydrite they can do more than simply adsorb to the surface of freshly precipitated ferrihydrite. Ligands, such as low-molecular-weight organic acids, may abate the degree of iron–oxygen and hydroxyl octahedra linking, diminishing the primary particle size and retarding maturation and crystallization of the cross-linked polymer of freshly precipitated ferrihydrite.38,39 We show here that simple aqueous conditions can be identified that allow small hydroxyl–carboxyl organic ligands to be co-precipitated with freshly forming ferrihydrite such that the ligand markedly disrupts ferrihydrite stability and, that in this fashion, bioavailable ferritin-core functional-mimetics were readily synthesised. For downstream applications (i.e. affordable supplementation) we focussed on cheap, generally recognized as safe (GRAS) dietary ligands to provide the modifications and we concentrated on nano-dispersion and enhanced rates of acid dissolution as initial desirable properties in light of current knowledge on dietary ferritin as an oral iron source.

Methods

Synthesis of solid ferrihydrite materials

The ligand-modified ferrihydrite materials were produced by co-precipitation and ligand substitution of oxo-hydroxy groups following the protocol described by Powell et al.38 Briefly, an acidic concentrated stock solution of Fe(III) chloride was added to a solution containing the ligand, buffer and 0.9% (wt/vol) of electrolyte (sodium chloride; NaCl) or, in the case of synthetic ferrihydrite, simply 0.9% NaCl. The initial pH of the mixture was always between 2.0, and the iron was fully solubilized. The pH was then slowly increased by drop-wise addition of concentrated solution of NaOH with constant agitation until reaching the desired final pH (ca. 7.4 for ligand-modified ferrihydrite and 7.4-8.2 for synthetic ferrihydrite). In the case of synthetic ferrihydrite the entire mixture was then oven dried at 45 °C for a minimum of 24 h. For the ligand-modified ferrihydrite materials, ultrafiltration (3,000 Da MWCO, Vivaspin®) followed by one H2OUPH wash was used to remove excess soluble ligand and buffer, i.e. non-bound or non-adsorbed to the nanoparticles (≥ 95% recovery according to the manufacturer’s specifications), before the material was oven dried (again 45 °C) for at least 24 h.

Physicochemical characterization

Iron content and phase distribution

Total iron content was determined by inductively coupled plasma optical emission spectrometry (ICP-OES JY 2000, Horiba Jobin Yvon Ltd., Stanmore, UK). ICP-OES standards and samples were diluted in 5% HNO3 to concentrations in the range 0-1000 ppm. Fractionation of the Fe into percentages of precipitated, nanoparticulate, and soluble Fe, for each of the Fe materials was achieved by centrifugation and ultrafiltration. Aqueous suspensions were centrifuged (10,000 × g, 5 min) and the sediment taken to be the precipitated fraction. In order to isolate the soluble Fe, and to distinguish it from disperse or colloidal (i.e. nanoparticulate) Fe, the supernatant was ultra-filtered (3,000 Da MWCO; 10,000 × g, 10 min). The Fe content
of all fractions (total, supernatant and ultrafiltrate) was determined by ICP-OES and expressed as percentage ± SD in relation to total Fe content as follows:

\[
\text{[(\% Fe precipitated)] = \left(\frac{(\text{Total Fe} - \text{Fe}_{\text{supernatant}})}{\text{Total Fe}}\right) \times 100}
\]

\[
\text{[(\% Fe nanoparticulate)] = \left(\frac{(\text{Fe}_{\text{supernatant}} - \text{Fe}_{\text{ultrafiltrate}})}{\text{Total Fe}}\right) \times 100}
\]

\[
\text{[(\% Fe soluble)] = \left(\frac{\text{Fe}_{\text{ultrafiltrate}}}{\text{Total Fe}}\right) \times 100}
\]

The iron materials were further characterized in terms of particle morphology by high-angle annular dark-field aberration-corrected scanning transmission electron microscopy (HAADF-STEM), X-ray diffraction, Fourier transform infrared spectroscopy (FTIR), electron energy-loss spectroscopy (EELS) and acid dissolution profiles. These methods are detailed in Supplementary Materials.

**Animal studies**

Six-week-old male mice, CD1 strain (Charles Rivers, Kent, UK), were used for the “ferrozine” study. Mice were housed in a light- and temperature-controlled room with ad libitum access to standard pellet diet and water. Animal care and the regulation of scientific procedures met the criteria laid down by the United Kingdom Animals (Scientific Procedures) Act 1986. Iron deficiency was induced by feeding 3- to 4-week-old mice a purified low-iron diet (Harlan Teklad, USA) with less than 1 mg Fe/kg for at least 2 weeks. Iron absorption studies were performed on iron-deficient mice fasted for ca. 16 h prior to the oral Fe dose. The Fe materials were prepared as described above at 20 mM Fe, labeled with \(^{59}\text{Fe}\) (ca. 3.1 MBq) at the time of synthesis (intrinsic labeling), and administered by oral gavage directly into the stomach as a single 100-μl dosage. Two oral doses of 10 mM ferrozine\(^{40}\) or water (control) were given 1 h prior to and during the gavage with the Fe compounds. Mice were left for 4 h with free access to drinking water until sacrifice and tissue collection. The stomach, small intestine and cecum plus colon were removed. The duodenum was removed and rinsed with 0.15 M NaCl. Intestinal tissue samples and duodenal washings were counted for \(^{59}\text{Fe}\) using a LKB Wallac gamma counter. The carcass was counted for \(^{59}\text{Fe}\) in a small animal whole body gamma counter (LIVE-1, Technical Associates, Canoga Park, CA). Standards were also measured in both counters to correct for the difference in sensitivity. Mucosal transfer was the amount of \(^{59}\text{Fe}\) in the carcass expressed as a percentage of administered dose; i.e. the amount of \(^{59}\text{Fe}\) radioactivity in the carcass 4 h after the gavage and is expressed as percentage of the total amount of radioactivity that is emptied out of the stomach. The soluble ferric iron control was Fe(III) nitrilotriacetate complex (i.e. Fe(III) NTA).

The bioavailability study in rats was conducted by MPI Research (Michigan, USA) and was approved by the Institutional Animal Care and Use Committee (IACUC) (MPI study protocol number 1925-001) following United States Animal Welfare Regulations. Hemoglobin was measured by clinical pathology as part of a complete blood count (CBC) panel. The increase in hemoglobin at day 14 was used as a measure of the bioavailability of the ligand-modified ferrihydrite in comparison with synthetic ferrihydrite and the current gold standard for iron supplementation (Fe(II) sulphate). Male Sprague–Dawley [Crl:CD\(^{®}\) (SD)] rats (n = 32) were approximately 21 days old at arrival (Charles River Laboratories). The animals were housed individually in polyboxes with toys and fed Block Lab Diet\(^{®}\) Certified Rodent Diet #5002 (PMI Nutrition International, Inc), ad libitum during the acclimation period (not less than 7 days). The control diet, i.e. the iron-deficient (ID) diet (AIN-93G Purified Rodent Diet, Dyets Inc., PA, USA) was administered to all animals from day 0 to day 24 (for the complete diet composition see Supplementary Table 2). The incorporation of the iron materials into the diets (~30 mg Fe/kg\(^{\text{diet}}\) and pelleting through extrusion were carried out by Dyets Inc. Other than the varying Fe compound, the diets were equivalent and conformed to AIN-93G-purified rodent diet.\(^{41}\)

Administration of the test diets, ID diet fortified with Fe(II) sulphate, ID diet fortified with synthetic ferrihydride, and ID diet fortified with tartrate-modified ferrihydrite, began on day 25. Three groups of eight male rats each were administered the test diets. One additional group of eight animals served as the no-iron control and continued to receive the un-supplemented ID diet. The test and control diets were administered for 14 days (i.e. until day 38). Rats consumed tap water and the control or test diets ad libitum throughout the study. Body weights were measured and recorded weekly. Blood samples for clinical chemistry evaluations were collected on days −1, 25, and 39. After the study termination (day 39) the rats were anaesthetized by CO\(_2\) inhalation and animals were euthanized by exsanguination.

**Results**

**Iron material characterization**

With initial screening in vitro we sought common GRAS (or “biologically safe”) ligands that would enable the formation of nano-dispersed, rather than aggregated or agglomerated, ferrihydrite materials upon titration of an acidic Fe(III)/ligand solution with base.

To ensure repeatability we used two separate buffers in parallel, namely adipate and tryptophan. Consistently, 40 mM Fe(III) and sub-equimolar tartrate yielded nano-dispersed Fe oxo-hydroxide materials: for optimal yield of the nano-disperse material the Fe(III)/tartrate ratio was 2:1 and pH buffered to greater than 7.0 (≥90% yield: open triangles, Figure 1, A and Supplementary Figure S1A). Buffer alone (adipate or tryptophan) had no effect and Fe(III) oxo-hydroxide aggregation and precipitation preceded as normal (closed circles, Figure 1, A and Supplementary Figure S1A).

We aimed to obtain dry materials that would re-disperse once aquated at the appropriate pH so, we next confirmed that, when re-introduced into the original volume of aqueous solution, the material again was nano-dispersed or precipitated according to pH in line with the original titration profile.
Precipitated (i.e. agglomerated) iron, determined following centrifugation (inset). Centrifugation and ultrafiltration to remove soluble iron (main panel), and of pH.

Figure 1. Formation of tartrate-modified ferrihydrite in adipate buffer as a function of pH. (A) Dispersed or colloidal (i.e. nanoparticulate) iron, determined following centrifugation and ultrafiltration to remove soluble iron (main panel), and precipitated (i.e. agglomerated) iron, determined following centrifugation (inset). Closed squares show synthetic ferrihydrite precipitated from an Fe(III) chloride solution; open triangles show modified ferrihydrite materials (molar ratios as above) re-suspended in the original volume of aqueous solution. (A and B) All values are expressed as a percentage of total iron in the initial solution as described in Methods. (C) Simulated gastric dissolution at pH 1.0 of dried synthetic ferrihydrite (closed squares) and tartrate-modified ferrihydrite (open triangles), at molar ratios as above, and then dried. Data were obtained following 5-min ultrafiltration (3000 Da MWCO); data are mean ± SD, n = 3.

Transmission electron microscopy (TEM) with electron energy-loss spectroscopy (EELS) was used to probe the local iron environments of the ferrihydrite materials (Figure 4). The Fe...
The $L_{2,3}$-edge spectrum of the synthetic (unmodified) material was again consistent with that of synthetic ferrihydrite\textsuperscript{27,46,47} while the tartrate-modified ferrihydrite Fe spectrum was almost identical suggesting that the ferrihydrite (or at least the Fe(III)) nature of the material was retained (Figure 4). An apparent very minor shift of the Fe $L_3$-peak maxima from 709.5 eV (Fe(III)) toward 707.5 eV (Fe(II)) should be viewed with caution as our previous data have shown that even with carefully controlled electron exposure, organic moieties will enhance the susceptibility of ferrihydrite to irradiation damage.\textsuperscript{27,46,47} The same should be noted for the carbon and oxygen $K$-edges at around 285 and 530 eV respectively, although the modified material shows, as expected, a much greater content of the organic-derived signals (Figure 4).

Taken together the XRD, STEM, FTIR and EELS data show that the tartrate-modified ferrihydrite is a disrupted or strained ferrihydrite structure where the tartrate ligand has been incorporated into the particles during co-precipitation and we suggest that this leads to a more acid-labile structure (Figures 2 and 4). Additionally, our data are also consistent with ligand bonding, between the tartrate, with its moderate affinity for iron (log $K_{\text{eff}} = 6.49$ at pH 3.54, 20 °C),\textsuperscript{48,49} and the surface of the ferrihydrite nanoparticles, so inhibiting further crystallization and growth\textsuperscript{39} and allowing nano-dispersion in aerated systems (Figure 1). Bioavailability

Therapeutic supplementation with oral Fe(II) salts (e.g. ferrous sulphate) induces significant gastrointestinal side effects in ~30% of subjects affecting compliance and thus effectiveness.\textsuperscript{50,51} This issue is considered, mostly to relate to iron redox cycling in the gut lumen leading to the generation of reactive oxygen species.\textsuperscript{52} Iron (III) salts are less prone to the initiation of redox cycling in this environment but are insoluble at intestinal pH and thus are poorly absorbed.\textsuperscript{53} Iron (III) chelates overcome the solubility issue but are expensive compared to salts and still undergo reduction at the mucosal surface, probably via duodenal cytochrome B.\textsuperscript{54} Here we show that when given by oral gavage to mice, tartrate-modified ferrihydrite is absorbed as efficiently as the Fe(III) chelate, ferric nitritolriacetate (Fe(III)NTA) (Figure 5, A). However, when the lumen of the gut was first flooded with ferrozine, an Fe(II) chelator that traps the luminal/mucosal surface-reduced iron,\textsuperscript{40} there was no impact on absorption of the tartrate-modified ferrihydrite, whereas Fe(III)NTA absorption was almost completely inhibited, confirming Fe(III)NTA requirement for reduction prior to uptake, and in contrary to that of the modified ferrihydrite (Figure 5, A).
therapeutic iron preparations. Overall, however, in such beneficial use in the production of both intravenous and oral agglomeration and aggregation, a property that has found polymeric sugars, the surface coat acts to prevent secondary trapping in, as well as adsorbed on, precipitating ferrihydrite. This might enable the organic acid to become meta-stable, crystalline phase. For large molecules, such as small anions or ligands, may enhance the formation of adsorption onto the freshly formed oxo-hydroxide nanoparticles. This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This might enable the organic acid to become meta-stable, crystalline phase. For large molecules, such as small anions or ligands, may enhance the formation of adsorption onto the freshly formed oxo-hydroxide nanoparticles. This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite (Figure 5, B). Finally, in iron-deficient anemic rats, tartrate-modified ferrihydrite, added to a standard diet at 30 mg Fe/kg diet, was able to restore hemoglobin levels over 2 weeks, in line with that of the gold standard ferrous sulphate (Figure 5, B). Synthetic ferrihydrite, added to the diet, had no impact on hemoglobin levels added to the same diet, at the same dose (Figure 5, B). There was no significant difference in the body weights of the animals on the tartrate-modified ferrihydrite supplemented diet in comparison with those on the diet supplemented with ferrous sulphate (Supplementary Table S1).

Discussion

Co-precipitation of metal ion hydroxides and organic moieties to form metal oxo-hydroxides with a degree of modification is well established. Typically this involves organic adsorption onto the freshly formed oxo-hydroxide nanoparticles which, for small anions or ligands, may enhance the formation of a meta-stable, crystalline phase. For large molecules, such as polymeric sugars, the surface coat acts to prevent secondary agglomeration and aggregation, a property that has found beneficial use in the production of both intravenous and oral therapeutic iron preparations. Overall, however, in such studies the primary particle (crystallite) of the metal oxo-hydroxide appears pure in its internal structure. In contrast, here we demonstrate that the polymeric, evolving primary particle of ferrihydrite, can be rendered impure or “doped” with a low-molecular-weight organic acid under specific conditions of co-precipitation. For tartrate the spacing between carboxyl and OH pairs that Fe ions might bind to during complexation in solution is similar to the distance between Fe atoms in partially ordered ferrihydrite. This might enable the organic acid to become trapped in, as well as adsorbed on, precipitating ferrihydrite particles. Certainly, if hydrolysis proceeds too rapidly the organic acid ligand fails to be incorporated within the evolving ferrihydrite particles and, instead, is simply adsorbed onto the primary particles. At the other extreme, excessive concentration of ligand may inhibit sufficient hydrolysis of iron. However, at appropriate ratios of Fe/ligand/OH, ligand-modified ferrihydrite can be achieved, the properties of which differ substantially to the pure synthetic oxo-hydroxides due to marked changes in crystallinity, surface charge and stability.

Our data are consistent with the recent studies of Eusterhues et al. who demonstrated that organic ligands may indeed modify, through inclusion, and disruption of iron octahedral cross-linking, the inter-atomic bond distances (d-spacings) and particle size of ferrihydrite. More recently, Mikutta has shown, with similar iron/ligand co-precipitation studies, that hydroxyl-benzoic acids may trigger the formation of small ferrihydrite nanoparticles with increased structural strain, but without altered d-spacing, in equilibrium with a fraction of lower-molecular-weight Fe(III) hydroxybenzoate complexes. In our system, however, the ligand-induced strain is sufficient to alter d-spacing (Figure 2). Furthermore, infrared analysis of ultrafiltered and washed material, obtained following repeated centrifugation steps, demonstrates co-association of ferrihydrite and bound tartrate but not buffer, again consistent with inclusion of tartrate in the ferrihydrite particles (Figure 3). Taken together our studies indicate that the active material reported herein is a ligand-modified form of ferrihydrite and not simply a mixture of small ferrihydrite nanoparticles and low-molecular-weight iron complexes. Indeed, dissolution studies indicate that all of the iron, and not just a labile fraction, is rendered soluble under lysosomal conditions (Figure 1, C). Secondly, ferrozine fails, entirely, to block intestinal uptake of the iron from tartrate-modified ferrihydrite (Figure 5, A). This again confirms that there is no labile Fe(III) fraction associated with the tartrate-modified ferrihydrite. Ferrozine is an Fe(II) chelator that has been well shown, both in vivo and in vitro, to markedly inhibit iron absorption from soluble Fe(III) complexes because these have an absolute requirement for reduction prior to absorption. Ferrozine traps the reduced iron (i.e. Fe(II)) and prevents iron absorption but no such effect was seen here with tartrate-modified ferrihydrite. Thirdly, our recent cellular studies with electron microscopy and inhibition of cellular endocytosis, demonstrate significant intestinal cell uptake of whole nanoparticles.

The aims of this work were not to mimic the ferrihydrite-like core of ferritin in appearance or molecular detail but, rather, in terms of its functional properties that allow for iron bioavailability following ingestion by humans: namely, nanoparticle dispersion and an enhanced rate of acid-induced dissolution (and, presumably, chelator-induced dissolution). Phosphate, for example, which is clearly involved in interactions between the ferritin protein and its ferrihydrite-like core, probably contributes to core instability and can certainly alter the structure of ferrihydrite following co-precipitation and hydrothermal annealing. However, in our work, additional phosphate ions provided no further effect over tartrate alone in destabilizing synthetic ferrihydrite and, when used instead of organic acid ligand, phosphate was ineffective (data not shown). The
additional factor of the protein shell in ferritin, as opposed to the protein-free structures reported herein, may explain differing phosphate sensitivities. In fact, the ligand-destabilized synthetic ferrihydrite of this report may even better mimic dietary Fe(III) that is dissolved under acidic, gastric conditions but then re-precipitates under the much less acidic intestinal conditions: endogenous ligands abound in the gastrointestinal lumen and soluble mucin (once referred to as gastroferrin)\textsuperscript{70} maintains the ferrihydrite nano-disperse.\textsuperscript{71} It is noteworthy that, systematically, the lysosome is used almost exclusively as the safe environment for macromolecular iron turnover (e.g. cycling and re-cycling of hemoglobin, Fe transferrin and ferritin) so it is not inconceivable that nanoparticulate iron is also acquired by the gut through intestinal endocytosis and then dissolved by lysosomal acid and ligands.\textsuperscript{31,72}

In the pursuit of novel, safe iron fortificants, Hilty et al.\textsuperscript{73} also demonstrated that nano sized Fe(III)-based particles are more bioavailable than bulk forms and are handled safely by the gut without contributing to abnormal tissue loading, although these authors regarded slow-release, gastric acid dissolution as an explanation for bioavailability of their Fe(III) nanomaterials. Whether dissolved in the gastric acid environment or the intestinal enterocyte lysosome, we concur with Hilty et al.\textsuperscript{73} that labile forms of nanosized Fe(III) would join the common (dietary) iron pool before basolateral export from the enterocyte, ensuring normal homeostatic control of these forms of iron. Moreover, for these nanomaterials the residual unabsorbed Fe(III) should transit the remaining gastrointestinal tract in a relatively safe, non-redox active form whether it be as the original nanostructure or re-precipitated following gastric emptying. Unlike previously reported nano Fe(III) materials, produced by flame pyrolysis\textsuperscript{42,73} and with organoleptic properties suitable for fortification\textsuperscript{a}, our approach focuses on cheap, GRAS reagents and facile synthesis to enable inexpensive but safe and effective supplementation\textsuperscript{a} which, as the World Health Organisation has noted, is especially required in developing and underdeveloped countries.\textsuperscript{1}

Overall, the ligand-modified ferrihydrite materials reported herein are noteworthy for their very small primary particle size (<5 nm) and enlarged lattice (circa 2.7 Å for the main Bragg peak) compared to synthetic ferrihydrite (2.6 Å). These findings therefore suggest that with appropriate buffers and Fe(III)/ligand/OH\textsuperscript{−} ratios, conditions can be achieved for ligand inclusion into the developing ferrihydrite particles, retarding both growth and crystallization, and favoring stabilization of the cross-linked polymeric structure. Such materials could have beneficial application in one key global health priority, namely the prevention and treatment of iron deficiency anemia without side effect-inducing redox cycling in the gastrointestinal tract.

\textsuperscript{a} Iron fortificants are added to foods whereas iron supplements are taken as oral tablets or capsules and typically at higher iron doses than for fortificants. The materials reported herein are suitable as supplements due to cheap manufacturing costs but lack the organoleptic properties (typically are too dark in color) for fortificants.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2013.12.011.

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