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Physico-chemical properties of the new generation IV iron preparations ferumoxytol, iron isomaltoside 1,000 and ferric carboxymaltose

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23

24 **Abstract**

25 The advantage of the new generation IV iron preparations ferric carboxymaltose (FCM),
26 ferumoxytol (FMX), and iron isomaltoside 1,000 (IIM) is that they can be administered in
27 relatively high doses in a short period of time. We investigated the physico-chemical properties
28 of these preparations and compared them with those of the older preparations iron sucrose (IS),
29 sodium ferric gluconate (SFG), and low molecular weight iron dextran (LMWID). Mössbauer
30 spectroscopy, X-ray diffraction, and Fe K-edge X-ray absorption near edge structure
31 spectroscopy indicated akaganeite structures (β -FeOOH) for the cores of FCM, IIM and IS, and
32 a maghemite (γ -Fe₂O₃) structure for that of FMX. Nuclear magnetic resonance studies confirmed
33 the structure of the carbohydrate of FMX as a reduced, carboxymethylated, low molecular
34 weight dextran, and that of IIM as a reduced Dextran 1,000. Polarography yielded significantly
35 different fingerprints of the investigated compounds. Reductive degradation kinetics of FMX was
36 faster than that of FCM and IIM, which is in contrast to the high stability of FMX towards acid
37 degradation. The labile iron content, i.e. the amount of iron that is only weakly bound in the
38 polynuclear iron core, was assessed by a qualitative test that confirmed decreasing labile iron
39 contents in the order SFG $\approx$ IS > LMWID $\geq$ FMX $\approx$ IIM $\approx$ FCM. The presented data are a step
40 forward in the characterization of these non-biological complex drugs, which is a prerequisite to
41 understand their cellular uptake mechanisms and the relationship between the structure and
42 physiological safety as well as efficacy of these complexes.

43

44 **Keywords**

45 Intravenous iron; iron sucrose; ferric carboxymaltose; iron isomaltoside 1,000; ferumoxytol; low
46 molecular weight iron dextran; NMR; XRD; Mössbauer; Fe K-edge XANES

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48

49 **Abbreviations**

50 DQF-COSY, double quantum filtered correlation spectroscopy; EDTA,
51 ethylenediaminetetraacetic acid; FCM, ferric carboxymaltose; FDA, U.S. Food and Drug
52 Administration; FMX, ferumoxytol; GFC, gel-filtration chromatography; Glc, glucose; GOF,
53 goodness of fit; HMBC, heteronuclear multiple-bond correlation; HMWID, high molecular weight
54 iron dextran; HSQC, heteronuclear single quantum correlation; IIM, iron isomaltoside 1,000; IS,
55 iron sucrose; ISS, iron sucrose similar; IV, intravenous; LMWID, low molecular weight iron
56 dextran; M_n , number average molecular weight; M_w , weight average molecular weight; M_z , z-
57 average molecular weight; NBCD, non-biological complex drugs; NMR, nuclear magnetic
58 resonance; NTBI, non-transferrin bound iron; P, polydispersity; PSC, polyglucose sorbitol
59 carboxymethylether; QS, quadrupole splitting; s, standard deviation; SAED, selected area
60 electron diffraction; SD_{CM} , carboxymethylation substitution degree; SFG, sodium ferric
61 gluconate; SHE, standard hydrogen electrode; NMR, transmission electron microscopy; TOCSY;
62 total correlation spectroscopy; TRIS, tris(hydroxymethyl)aminomethane; USP, United States
63 Pharmacopeia; XANES, X-ray absorption near edge structure; XRD, X-ray diffraction

64

65 **Introduction**

66 Intravenous (IV) iron therapy is widely used to treat iron deficiency and iron deficiency anemia
67 (Auerbach and Ballard 2010). The indications include absolute iron deficiency, when there is a
68 need for fast iron repletion or an intolerance to oral iron, as well as the therapy of anemia of
69 chronic disease (ACD, also called iron sequestration syndromes) or functional iron deficiency
70 (Goodnough et al 2010). Under inflammatory conditions (anemia of chronic disease) or when
71 there is a high iron demand for erythropoiesis (functional iron deficiency), such as during therapy
72 with erythropoiesis stimulating agents, oral iron therapy is not effective and IV iron is
73 recommended (Goodnough et al 2010; Qunibi 2010). Therefore, the therapeutic areas for IV iron
74 are widespread and, among others, include nephrology (Besarab and Coyne 2010; Macdougall
75 et al 2012), cardiology (Avni et al 2012; Macdougall et al 2012; von Haehling et al 2012),
76 oncology (Gaftner-Gvili et al 2013), gastroenterology (Gomollon and Gisbert 2013), and
77 gynecology (Breyman et al 2010; Haththotuwa et al 2011), as well as patient blood
78 management (pre-/postoperative anemia) (Shander et al 2012).

All iron compounds used for IV iron therapy consist of a polynuclear Fe(III)-oxyhydroxide/oxide core surrounded by carbohydrates which stabilize the core and protect the nanoparticles against further polymerization (Auerbach and Ballard 2010; Kästele et al 2014; Macdougall and Geisser 2013; Qunibi 2010). Despite their similar structure, IV iron compounds have distinct properties. In particular, they display a wide range of stability and, depending on the type of stabilizing carbohydrate, may have antigenic potential (Chertow et al 2006). IV iron compounds belong to the class of so-called non-biological complex drugs (NBCD) which, in contrast to small molecules, cannot be fully characterised by physico-chemical methods and which are largely defined by the manufacturing process (Crommelin et al 2014).

A main goal of the recent developments in the field of IV iron therapy was to provide a preparation that can be administered in higher doses and in a short period of time. Some of the older products, such as sodium ferric gluconate (SFG) and iron sucrose (IS) are not very stable and thus contain a higher percentage of labile, weakly-bound iron (Van Wyck et al 2004; Van Wyck 2004). This property, together with the high osmolarity and the high pH of the solutions, limits the maximum single doses allowed for SFG and IS to 62.5–125 mg and 200–500 mg iron (Fe), respectively (Macdougall and Geisser 2013). In contrast, the three recently introduced IV iron preparations Ferinject®/Injectafer® (active ingredient: ferric carboxymaltose, FCM), Feraheme®/Rienso® (active ingredient: ferumoxytol, FMX), and MonoFer® (active ingredient: iron isomaltoside 1000, IIM), are more stable and can all be administered in comparatively high single doses (from 510 up to more than 1,000 mg Fe) (Macdougall and Geisser 2013). An overview on selected properties and recommended dose regimen of the different preparations is given in Table 1.

FCM consists of a polynuclear Fe(III)-oxyhydroxide core surrounded by carboxymaltose which is derived from maltodextrin, an oligosaccharide produced from starch. IIM contains a polynuclear Fe(III)-oxyhydroxyde core which is stabilized by a hydrogenated (reduced) Dextran 1,000 (isomaltoside 1,000) and a low amount of citrate (Andreasen and Christensen 2001; Medice Pharma GmbH&Co. KG, Iserlohn, Germany 2011; Nordfjeld et al 2012; Pharmacosmos A/S, Holbaek, Denmark 2009). A different, matrix-like structure has been proposed for IIM (Jahn et al 2011), but has recently been shown to be based on an incorrect interpretation of nuclear magnetic resonance (NMR) data (Kästele et al 2014). FMX was originally developed as a contrast agent for magnetic resonance imaging and has been described as a superparamagnetic ferric oxide coated with polyglucose sorbitol carboxymethylether (PSC), a reduced and carboxymethylated dextran (Simon et al 2006). FMX contains mannitol and further

PSC as excipients (AMAG Pharmaceuticals Inc., Waltham, MA, USA 2009; European Medicines Agency 2012).

In this work, the physico-chemical properties of these three new preparations for parenteral iron therapy were characterized and compared. In particular, we show that despite the similarity of the investigated compounds, they show unique properties and important differences which may have an implication for their therapeutic application. Comparable techniques have been used previously to characterize some of these compounds, but direct comparison is not always available and there is not always a good agreement among the results (Balakrishnan et al 2009; Funk et al 2001; Fütterer et al 2013; Jahn et al 2011; Kudasheva et al 2004).

Materials and methods

Materials

The following IV iron preparations were obtained from a pharmacy or directly from the manufacturer: Feraheme[®] (30 mg Fe/mL, AMAG Pharmaceuticals, Inc., Lexington, MA) and Rienso[®] (Takeda Global Research and Development Centre (Europe) Ltd., Aldwych-London, UK), active ingredient ferumoxytol (FMX); MonoFer[®] (100 mg Fe/mL, Pharmacosmos A/S, Holbaek, Denmark), active ingredient iron isomaltoside 1,000 (IIM); Ferinject[®] (50 mg Fe/mL, Vifor (International) Ltd., St. Gallen, Switzerland), active ingredient ferric carboxymaltose (FCM); Venofer[®] (20 mg Fe/mL, Vifor (International) Ltd., St. Gallen, Switzerland), active ingredient iron sucrose (IS); Cosmofer[®] (50 mg Fe/mL, TEVA GmbH, Radebeul, Germany), active ingredient iron dextran, and Ferrlecit[®] (12.5 mg Fe/mL, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany), active ingredient sodium ferric gluconate (SFG). Since different lots were used, the lot numbers are indicated for each method separately.

Separation of mannitol from the FMX solution

To remove mannitol, 18 mL FMX solution (Feraheme[®] lot 09060402) were diluted with 7.2 mL distilled water and filtered with an Amicon Ultra-15 centrifugal filter device (IVD ultracel-10K regenerated cellulose, molecular weight cut-off 10 kDa, Millipore, Ireland). The retentate was taken up six times with distilled water and centrifuged. The final retentate contained the mannitol-free FMX. Although the weight-average molecular weight of the carboxymethylated

dextran in FMX is above the molecular weight cut-off of the filter, low-molecular weight fractions of the unbound carboxymethylated dextran may also have been partly removed from the solution by this method. The resulting material was used for X-ray diffraction (XRD) measurements and as starting material for the isolation of the FMX carbohydrate.

Isolation of the carbohydrates from the FMX and IIM solutions

The iron-carbohydrate nanoparticles were destroyed by heat treatment of the mannitol-free FMX (see section above) and IIM (MonoFer[®] lot 949171-1) solutions in phosphate buffer: Product solution corresponding to 120 mg iron was mixed with 50 mL phosphate buffer (4.43 g KH₂PO₄/0.143 g Na₂HPO₄ per 500 mL) and heat treated at 132°C and pH 5.4 for 90 min. The iron phosphate precipitate was separated from the carbohydrate-containing supernatant by centrifugation (10 min at 500 U/min). The heat treatment and centrifugation was repeated with the supernatant (132°C, 15 min for FMX and 132°C, 90 min for IIM). The filtrate of the supernatant was lyophilized and dissolved in distilled water (1.17% [m/m]). The resulting carbohydrate-containing solution from FMX was dialyzed with a regenerated, ethylenediaminetetraacetic acid (EDTA)-treated cellulose membrane (nominal cut-off 1,000 Da, V-series by ZelluTrans Roth, Karlsruhe, Germany, lot 190119) to remove phosphate and again lyophilized. The lyophilisate from FMX most likely contains both the core-stabilizing PSC and PSC added as an excipient. Due to the lower molecular weight of isomaltoside 1,000, the carbohydrate-containing solution from IIM was not purified by dialysis but by ion exchange (Merck ion exchanger V, no. 104836. Lot L701436 101) to remove the phosphate.

Mössbauer spectroscopy

An aliquot of 400 µl (FMX and IIM) or 450 µl (FCM) of liquid sample material was pipetted under oxic conditions into a Teflon sample container, sealed with Kapton tape and frozen at –30°C. The frozen samples were transferred into a closed cycle helium cryostat and Mössbauer spectra were recorded at temperatures between 245 and 5 K. The Mössbauer spectra were recorded in transmission geometry using a constant acceleration drive system equipped with a ⁵⁷Co source in rhodium matrix and a proportional counter coupled to a CMCA-550 1024 multichannel analyzer (WissEL, Germany). All spectra were calibrated against a room temperature spectrum of a 7 µm alpha-Fe foil. The spectra were fitted using Voigt based spectral lines (RECOIL

software suite, University of Ottawa, Canada). During fitting, the half-width-half-maximum of the peaks was kept at 0.097 mm/s and the Gauss sigma parameter was varied to account for line broadening. The blocking temperature was defined as the temperature at which 50% of the material was paramagnetic and 50% magnetically ordered (Funk et al 2001; Murad 1996). The following lots were analyzed: MonoFer[®] lot 949171-1, Feraheme[®] lot 09060402, Ferinject[®] lot 062201.

Iron(II) quantification

The amount of Fe(II) was determined by cerimetric titration with cerium sulfate and potentiometric end-point determination (Jander et al 2003). The following lots were analyzed: MonoFer[®] lot 949171-1, Feraheme[®] lot 09060402, and 9 consecutive lots of Ferinject[®].

X-ray diffraction (XRD)

The samples were measured on different instruments (Bruker, Karlsruhe, Germany) as indicated in the Supplementary data. All samples were transferred to a flat disk sample holder with a 1 mm deep round indentation with 20 mm in diameter. α -Al₂O₃ (corundum) was applied as an internal standard to all samples. Liquid samples were applied onto a Si single-crystal chip (diameter 1 inch) and air-dried at 50°C.

The diffractograms were analyzed with the softwares DiffracPlus EVA 15.0 for a first identification of the crystalline phases, and TOPAS 4-2 (both Bruker AXS GmbH, Karlsruhe, Germany) for a more advanced determination of lattice constants by the Pawley method (Pawley 1981). Space group information was obtained from literature. The profile function applied to all refinements was a modified Thompson-Cox-Hastings pseudo-Voigt function (Young 1993). The goodness of fit parameter (GOF) was also calculated. A GOF of 1.0 corresponds to a perfect agreement between model and data. All programs were provided by Bruker AXS (Karlsruhe, Germany). The domain size was determined from the Scherrer equation (Klug and Alexander 1974) and consideration of the instrumental contribution to line broadening determined from LaB₆ measurements.

X-ray absorption near edge structure spectroscopy (XANES)

Fe K-edge XANES spectroscopy was performed at the X-ray beamline of the Synchrotron Radiation Laboratory for Environmental Studies (SUL-X) of ANKA (Karlsruhe Institute for Technology, Eggenstein-Leopoldshafen, Germany) using a Si(111) crystal pair in a fixed exit monochromator. To avoid radiation damage the beam was collimated. Pellets of fine grained goethite (α -FeOOH), lepidocrocite (γ -FeOOH), 2-line and 6-line ferrihydrite, as well as akaganeite (β -FeOOH) were prepared with cellulose in order to achieve optimal absorption for the spectra that were used as references. The liquid sample solutions (MonoFer[®] lot 224121-3; Venofer[®] lot 076201) were measured in a custom-made cell for liquid samples. The cell mainly consists of two Kapton-foils with an effective diameter of 10 mm and 0.7 mm separation, which results in a volume of 0.55 mm³. The foils are sealed with O-rings, and the parts are kept together by a metallic frame equipped with in- and outlets. The 0.7 mm spacing has been chosen because at that distance the transmission signal is not disturbed by the undiluted sample solution. Two cell fillings of each substance were measured, and each measurement was performed twice at the same spot to detect instrumental influences or changes within the sample that may occur due to high photon flux density. Energy step width across the edge was 0.3 eV. The energy has been calibrated to 7112 eV at the first maximum of the first derivative of a XANES spectrum of elemental Fe (Fe foil). All measurements were done in transmission mode using ionization chambers as detectors for the incident and absorbed beams of sample, reference substances and Fe foil. Spectra were added and averaged. Their pre- and post-edge ranges were approximated by linear and polynomial fit-functions. The edge jump was normalized to 1 in order to compare spectra with each other. Both steps of data processing were done with the Athena program of the IFEFFIT package (RAVEL and Newville 2005). Combinatorial Linear Combination Fits (LCF) have been performed with the Athena program of the IFEFFIT package for the Fe K-edge XANES spectra with five reference spectra: akaganeite, goethite, lepidocrocite, 2L-ferrihydrite (2L-Fh) and 6L-ferrihydrite (6L-Fh) (RAVEL and Newville 2005).

NMR spectroscopy

The ¹H and ¹³C NMR spectra of the IIM and FMX carbohydrates were recorded at 400.13 and 100.61 MHz on a Bruker Avance 400 NMR spectrometer (Bruker Biospin AG, Fällanden, Switzerland). The ¹H, the 1D diffusion-edited ¹H, and the ¹³C NMR spectra, as well as the ¹H-¹³C HSQC, ¹H-¹³C HMBC, ¹H-¹³C HSQC-TOCSY, and ¹H-¹H DQF-COSY 2D correlation NMR experiments were performed at 298 K on a 5 mm broadband inverse probe equipped with z-gradient (100% gradient strength of 53.5 Gcm⁻¹) applying 90° pulse lengths of 6.8 μ s (¹H) and

14.5 μs (^{13}C). All NMR experiments were performed in D_2O solutions ($c = 50\text{--}200\text{ mg/mL}$) using the Bruker standard pulse programs and parameter sets selecting coupling constants of 145 Hz (HSQC), 10 Hz (HMBC) and mixing times of 150 ms (HSQC-TOCSY). The ^1H and ^{13}C chemical shifts were externally referenced relative to the signals of 3-trimethylsilyl-2,2,3,3-tetradeutero sodium propionate dissolved in D_2O at 0.0 and -1.6 ppm, respectively. The carbohydrates of following lots were analyzed: MonoFer[®] lot 949171-1, Feraheme[®] lot 09060402 and lot 10012802; Dextran 3–4,000 (Amersham Biosciences, lot 303820) and Dextran 1,000 (Sigma-Aldrich Switzerland, Lot BCB4347V-02-002) were used as reference samples.

Molecular weight distribution

The molecular weight distribution was determined by gel-filtration chromatography (GFC) as described earlier (Geisser et al 1992). The lot numbers of the investigated samples are given in the results section. For the determination of the molecular weight of carbohydrate samples, glucose and Dextran 1,000 were used in addition to the pullulans P5, P10, and P20 as calibration standards (Geisser et al 1992).

Polarographic analysis

The reduction potentials were measured by differential pulse polarography based on the US Pharmacopeia (USP) method described in the monograph for iron sucrose injection (U.S. Food and Drug Administration 2012b). The measurements were done at pH 7 in 15% m/v acetate buffered solutions on a Metrohm 797 VA Computrace polarograph (Metrohm AG, Herisau, Switzerland) with a multi-mode working electrode, reference electrode $\text{Ag/AgCl}/c(\text{KCl})=1\text{ mol/L}$, and with a platinum auxiliary electrode. The values reported in this work have been corrected by +236 mV to express the potentials relative to the standard hydrogen electrode (SHE). The iron concentrations in the sample solutions were 15 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 20 $\mu\text{g/mL}$, and 25 $\mu\text{g/mL}$ for FMX, IIM, IS, and FCM, respectively. All iron formulations were measured immediately after opening the container. The following lots were analyzed: Feraheme[®] lot 10061002, MonoFer[®] lot 042838-3, Venofer[®] lot 901201, Ferinject[®] lot 169001.

Reductive degradation kinetics

The reductive degradation kinetics were measured at pH 2.50–2.60 and 25°C in solutions containing ascorbic acid, citric acid, H₃PO₄, Na₂HPO₄ (all 0.08 M), FeSO₄ (0.008 M), and sorbitol (1 M) (Erni et al 1984). The analyzed lots are given in the results section.

“Tea test”

The "tea test" was used as a qualitative assay to visualize the content of labile, weakly-bound iron by reaction with polyphenols. Five bags of Lipton white tea (lot L23320D023; exp 11/2014. Unilever Schweiz, 8240 Thayingen, Switzerland) were placed in 2 L of a 0.9% NaCl solution at 90–100°C and allowed to steep for 1 minute. White tea was chosen because of its light color combined with a high content of polyphenols. After the infusion had cooled to <37°C, the iron preparations were added to result in an iron concentration of 0.1 mg/mL and stirred (Ferinject[®] lot 258001; MonoFer[®] lot 042838-3; Feraheme[®] lot A56996A; Venofer[®] lot 133001; Cosmofer[®] lot 1226801-3; Ferrlecit[®] lot D2A046A). No pH adjustment was done. The solutions were filled into cuvettes, and photographs were taken 1–2 hours after sample preparation had started.

281

282 **Results and discussion**

283 **Characterization of the polynuclear iron oxide/hydroxide cores**

284 *Mössbauer spectroscopy*

285 The Mössbauer spectra of FMX could be modeled with parameters characteristic for Fe(III), with
286 no indication of the presence of Fe(II). Based on hyperfine field parameters of the 5 K spectrum,
287 FMX was identified as nano-maghemite (Figure 1, Table 2) (Tronc et al 2000; Tucek et al 2006).
288 The transition from the superparamagnetic to the magnetically-ordered state spanned a wide
289 temperature range of more than 100 K. While the material is completely magnetically ordered at
290 5 K, first signs of magnetic relaxation were detected at 45 K. For higher temperatures an
291 increasing paramagnetic contribution was found. At 245 K, the superparamagnetic doublet was
292 still strongly broadened, showing that the material was not completely superparamagnetic
293 although no distinct sextet features were visible. The coexistence of doublet and sextet features
294 over a large temperature range has been described for nanoparticle suspensions of maghemite
295 (Tronc et al 2000). The magnetic blocking temperature of FMX was found to be 73 K. The
296 blocking temperature depends mainly on the particle size, crystal structure and interparticle
297 interactions. Based on the assignment as a maghemite suspension, the low blocking
298 temperature of FMX suggests that the iron cores are in the range of 5–10 nm (Morup and Tronc
299 1994; Tronc et al 2000).

300 The Mössbauer spectra of IIM could also be modeled with parameters characteristic for Fe(III),
301 with no indication of the presence of Fe(II). With an average hyperfine field of 47.6 T, IIM
302 showed a significantly weaker hyperfine field at 5 K than FMX (Table S2, Supplementary data).
303 Additionally, some magnetic relaxation, visible as innerline broadening, was present even at 5 K.
304 These spectral features suggest a low crystallinity of the iron cores. The hyperfine field of 47.6 T
305 combined with a center shift of 0.48 mm/s are indicative for an akaganeite structure (Barrero et
306 al 2006; Bigham et al 1990). The blocking temperature was 56 K (Table S2, Supplementary
307 data). The spectra between 30 and 60 K showed an increase in paramagnetic contribution, and,
308 at 77 K, 66±1.5% of the spectral area showed magnetically-ordered material (Figure 1). As
309 reported earlier (Jahn et al 2011), the material is completely paramagnetic at 150 K.

Also the spectra of FCM suggested an akaganeite-like structure without indication of the presence of Fe(II). A second sextet was necessary to model the spectra between 5 and 77 K. The two models used for the spectrum at 5 K had a similar average of the center shift and a slightly weaker magnetic hyperfine field than that of IIM (Figure 1; Table S2, Supplementary data). The hyperfine field strengths of the sextets were slightly smaller than expected for akaganeite. This could arise from iron on the surface of the mineral cores with a different binding environment compared to bulk material, defects in the akaganeite structure such as chloride vacancies or some additional lepidocrocite in the sample (Bigham et al 1990; Murad and Cashion 2004). Despite the visible magnetic relaxation at 5 K, which suggests a low crystallinity, the blocking temperature of 114 K was the highest of the three samples. Between 30 and 120 K, both magnetically-ordered material and superparamagnetic material were present in the sample, while at 245 K the sample was completely paramagnetic, lacking the broadening observed for FMX (data not shown).

3.1.2. X-Ray diffraction (XRD)

XRD diffractograms of FMX, recorded for the mannitol-free sample, allowed the identification of the FMX core as a maghemite (γ -Fe₂O₃ space group #96 P4₃2₁2) without any signals of magnetite (Figure 2a). Models of akaganeite and magnetite worsened the agreement between model and raw data. The best GOF found with maghemite was 6.8, which still represents a lower quality fit. The lattice constants (Table S3, Supplementary data) were in good agreement with reference data for maghemite (Pecharrroman et al 1995), and the domain sizes were determined to lie around 10 nm, which is in agreement with the Mössbauer data (5–10 nm) and close to the earlier reported core diameter of 6.4 nm (Jahn et al 2011).

The diffractogram of IS appeared feature-poor with broad peaks, which resulted in an arguable fit with the different possible crystallite structures. A mixture of goethite (α -FeOOH, space group #62 Pbnm) and akaganeite (β -FeOOH, space group 12 I2/m) could be postulated (calculated GOF value 1.2) (Figure 2b). Previously published XRD analyses were interpreted in a variety of different ways: the core structure of IS was assigned as a ferrihydrite (Funk et al 2001), a ferrihydrite with possibly other structures mixed in such as akaganeite (Jahn et al 2011), as a lepidocrocite or ferrihydrite (Fütterer et al 2013), or as akaganeite (Kudasheva et al 2004). The inconsistency of these results may partly arise from different experimental details such as the drying conditions. Moreover, a very small crystallite size or a low crystallinity in the IS core makes XRD, a technique suitable for the characterization of long-range order, not an ideal

method for the investigation of IS. In fact, earlier XRD results suggest a core diameter of between about 1 and 3 nm (Funk et al 2001; Jahn et al 2011; Kudasheva et al 2004).

The mineral core of FCM could clearly be identified as akaganeite (β -FeOOH, space group #12 I2/m) (Figure 2c; Table 2). The lattice constants (Table S3, Supplementary data) were in good agreement with literature data (Post and Buchwald 1991). The GOF reached 1.4, and the domain size for the akaganeite lay around 4–5 nm, which is in agreement with the value of 4.3 nm reported earlier (Jahn et al 2011).

The mineral core of IIM could most likely be attributed to an akaganeite-like phase (β -FeOOH, space group #12 I2/m) (Figure 2d; Table 2). Though accompanied by a large standard deviation, the calculated lattice constants (Table S3, Supplementary data) were in good agreement with reference data for akaganeite (Post and Buchwald 1991). The domain sizes derived were around 3 nm. These results agree well with the previously published description of the IIM core as being similar to akaganeite with a core diameter derived by XRD of 4.2 nm (Jahn et al 2011). As described for IS, also IIM showed the limitation of XRD for poorly crystalline and/or very small particles. The agreement between model and raw data was poor for all structures tested, which were goethite, lepidocrocite, maghemite and magnetite. The best GOF was obtained by an akaganeite structure with a value of 16.6. The high GOF value and the large variation in the difference curve reflect a low conformity between the applied model and the data, which was observed previously (Jahn et al 2011).

X-ray absorption near edge structure (XANES) spectroscopy

The relatively broad peaks in the XRD diffractograms and the high GOF values of the described substances show the limitation of XRD for poorly crystalline or very small particles, especially for IS and IIM. Fe K-edge XANES spectroscopy was chosen to better characterize IS and IIM, because it gives information about the short range order of an absorbing element, here Fe, and hence can be applied also for low- or non-crystalline materials. Furthermore, because Fe K-edge XANES measurements were done with liquid samples, artifacts from the drying process, which may play a role especially for the XRD of IS, can be excluded.

The XANES spectra with details of their pre-edge, main-peak and post edge energy ranges are shown in Figures 3a-d. It is evident that the curves of the two liquid samples of IIM and IS are deviating significantly from those of goethite, lepidocrocite and ferrihydrites. A good match is observed between the sample spectra and that of akaganeite. Linear combination fits (LCF)

confirm that the sample spectra can be described with a major fraction of akaganeite (see Supplementary data, section 2). In fact, the best fits are achieved with some ferrihydrite fraction (about 27% for IS and 17% for IIM), but do not result in better matches for certain XANES features like the top of the main peak and the pronounced shoulder at the high energy flank of the main peak (Figures S1a,b and S2a,b, Supplementary data). Hence, this result indicates that the short-range order structures of the iron-containing phase in IS and IIM are similar to akaganeite.

Iron(II) content

Cerimetric titration yielded Fe(II) concentrations relative to the total Fe content of 0.6% for IIM, 0.8% for FCM, and 1.0–1.3% for FMX. These results are consistent with the fact that no Fe(II) was detected in the Mössbauer spectra (detection limit of 1.5–2.5%) and in the Fe K-edge XANES spectra (detection limit >10%), and it also agrees with the identification of the FMX, IIM, and FCM core minerals as maghemite (FMX) and akaganeites (IIM, FCM), respectively.

Discussion of the iron core structures

The cores of a number of polynuclear Fe(III)-oxyhydroxide carbohydrate nanoparticles, i.e. iron dextran, iron dextrin, and iron polymaltose have been reported to have akaganeite structures (β -FeOOH) (Funk et al 2001). Among the new generation IV iron preparations, a nonstoichiometric magnetite structure (Coyne 2009), an XRD pattern close to that of magnetite and maghemite (Jahn et al 2011), later reassigned as pure magnetite (Fütterer et al 2013) have been reported for FMX, and an akaganeite structure has been described for FCM and IIM (Jahn et al 2011). Yet, in the same work (Jahn et al 2011), the IIM structure has also been described as a “matrix structure” based on ^{13}C NMR data. There seems to be some confusion in the literature about what exactly is meant with a “matrix structure” (Fütterer et al 2013; Jahn et al 2011). A very detailed model with the iron atoms chelated between the carbohydrate chains of the ligand has been presented earlier [figure 11 in (Jahn et al 2011)], whereas it was recently mentioned that the iron hydroxide is rather stable and this feature has been assigned to the matrix structure (Fütterer et al 2013). An in-depth NMR study on the carbohydrate–Fe(III) bonding situation in different IV iron preparations, however, disproved the chelation of iron and the matrix structure

for IIM, which was stated to derive from an incorrect interpretation of the NMR spectra (Kästele et al 2014).

The data reported in this work support akaganeite as the core structure of IIM and FCM as well as for IS. IIM showed a smaller crystallite size than FCM by XRD, which agrees with the wide diffraction lines in the XRD of IIM and the low blocking temperature found by Mössbauer spectroscopy. Earlier XRD data suggested approximately the same crystallite sizes for IIM and FCM of 4.2 and 4.3 nm, respectively (Jahn et al 2011), which is very close to the values of 3 and 4–5 nm reported here. The Mössbauer spectra of IIM and FCM show comparable hyperfine field parameters at 5 K which are most similar to the parameters of akaganeite. Both materials show some magnetic relaxation at 5 K which suggests a low crystallinity of the iron core. The differences in the magnetic blocking temperatures of IIM and FCM may be explained by a larger size of the iron core or by inter-particle magnetic interaction in FCM at low temperatures, a rationale that has also been given for nano-magnetite suspensions (Morup and Tronc 1994; Tucek et al 2006). By XRD, the lattice constants of IIM and FCM were also found to be very similar to each other and in good agreement with akaganeite, although the GOF of IIM was rather poor, probably due to its small particle size. Fe K-edge XANES spectra of IIM further confirmed the akaganeite structure of the IIM core. It can thus be concluded that IIM and FCM have akaganeite core structures, but the domain size of IIM is somewhat smaller than that of FCM (Table 2).

For FMX, Mössbauer and XRD data as well as the very low Fe(II) content indicate a maghemite core structure, which is in contrast to previous descriptions of the material as a (AMAG Pharmaceuticals Inc., Waltham, MA, USA 2009; Coyne 2009) magnetite or, also based on XRD investigations, as a structure close to that of magnetite and maghemite (Jahn et al 2011). In a follow-up paper, Langguth and coworkers (Fütterer et al 2013) reassigned the structure as pure magnetite based on selected area electron diffraction (SAED) data, by stating that the two structures can be differentiated based on missing rings and respective d-values in the SAED pattern. However, with the presented d-values from the SAED data one can calculate a lattice parameter of 0.844 ± 0.011 nm (space group #227), which does not correspond to magnetite or maghemite. This result is not unexpected, as electron diffraction in transmission electron microscopy (TEM) is not accurate enough to distinguish the minor differences between cubic maghemite (fully or nearly fully oxidized magnetite) and stoichiometric magnetite. In conclusion, a clear differentiation between magnetite and maghemite can only be done by more appropriate methods, e.g. by Mössbauer spectroscopy. The very low amount of Fe(II) found by cerimetric titration (1.0–1.3%) as well as the absence of Fe(II) by Mössbauer spectroscopy (detection limit

of 1.5–2.5%) support our assignment of the FMX core structure as maghemite. Finally, the red-brown color of the FMX-solution confirms the assignment as maghemite [pure Fe(III)], as the mixed-valent magnetite would be expected to be black (Cornell and Schwertmann 2003).

The domain size of FMX was approximately 10 nm and 5–10 nm, derived by XRD and Mössbauer spectroscopy, respectively, and, thus, was found to be larger than that of IIM (about 3 nm) or FCM (4–5 nm) (Table 2) and similar to the 6.4 nm reported earlier (Jahn et al 2011).

Characterization of the carbohydrate components of IIM and FMX by NMR and GFC

The structures of the carbohydrates isolated from FMX and IIM were identified by 1D and 2D ^1H and ^{13}C NMR investigations. Dextran 1,000, Dextran 3–4,000, reduced Dextran 3–4,000, and reduced and carboxymethylated Dextran 1,000 were used as reference materials. In addition to the characterization by NMR spectroscopy, the molecular weight distributions of the isolated carbohydrate components of IIM and FMX were investigated by GFC.

The carbohydrate component of IIM (isomaltoside 1,000) could be identified as a linear, low molecular weight, reduced Dextran 1,000 (Andreasen and Christensen 2001; Pharmacosmos A/S, Holbaek, Denmark 2009). The NMR spectra of isomaltoside 1,000 match the spectra of Dextran 1,000 very closely and provide proof for the α -(1→6)-linked glucan backbone of dextrans (Figure 4), but the signals from the reducing glycosides (species $\text{C}\alpha$ and $\text{C}\beta$) are almost completely absent (Figure 5; Figure S3, Supplementary data). The resonances of an additional $\text{CH}_2\text{-OH}$ group could be assigned to a reduced C-1 end unit (species C_{red} and C'_{red}), which shows that close to 100% of the C-1 ends were reduced (Figures 4, 5). This indicates that no major hydrolysis occurred during the isolation of the carbohydrate. No signs of branched units (1,3-br. or 1,3-br.') were detected in isomaltoside 1,000 or in Dextran 1,000. The NMR data show that isomaltoside 1,000 consists in average of 5 glycan units with a M_n of about 800 Da, which is slightly lower than the chain length of Dextran 1,000 from Sigma Aldrich with about 6–7 units and a M_n of about 1,100 (Table S5, Supplementary data). For isomaltoside 1,000, GFC yielded 1,020 Da and 790 Da for M_w and M_n , respectively (data not shown). The molecular weight distribution parameters obtained for isomaltoside 1,000 by GFC are in good agreement with the M_n values derived from NMR measurements and with published information (Andreasen and Christensen 2001; Pharmacosmos A/S, Holbaek, Denmark 2009).

The carbohydrate component of FMX (PSC) was identified as a dextran with a low degree of branching, partly carboxymethylated at positions C-2, C-3, or C-4 in the glucan backbone and with a reduced, non-carboxymethylated C-1 chain end unit. The resonances from species A in the glycoside chain and B at the non-reducing chain end are readily observed, indicating the α -(1 $\rightarrow$ 6)-linked glucan backbone, and a weak resonance at 5.33 ppm provides evidence for a low degree of branching of 1–2% (species 1,3-br) (Figure 5; Figure S3, Supplementary data). In agreement with the description of the FMX carbohydrate by the manufacturer as a polyglucose sorbitol carboxymethylether (European Medicines Agency 2012; Groman et al 2003) the C-2, C-3 and C-4 carboxymethylated species in the glycoside main chain could be identified by NMR (species D, E, and F). No evidence of carboxymethylation at the primary alcohol group of the C-6 chain end or at the reduced C-1 chain end was found. At the C-1 chain end, all ^1H and ^{13}C NMR resonances characteristic for species C_{red} (Figure 4) were detected, implying that the C-1 chain end was reduced but not carboxymethylated (Figure 5; Figure S3, Supplementary data). The identity of the carboxymethylated species D, E, F, and of species B and C_{red} at the glycoside chain ends were unambiguously identified by the NMR analysis of the reduced and carboxymethylated Dextran 1.

A quantitative evaluation of the NMR data suggested that PSC is composed of 20–22 units with a M_n of approximately 3,400–3,800 Da. The degree of carboxymethylation of the FMX carbohydrate was determined to lie around 0.18–0.23, which corresponds to one carboxymethylated unit for every 4–5 glycan units (Table S5, Supplementary data). The determination of the molecular weight distribution by GFC yielded a M_w of 14.2 kDa, a M_n of 8.2 kDa, and a polydispersity (P) of 1.73 (data not shown). The observed discrepancy between the M_n value obtained by GFC and that obtained by NMR is probably at least partly due to the higher excluded volume (i.e. lower apparent density) of a carboxymethylated dextran relative to that of a native dextran.

It is conceivable that the conditions applied for the isolation of the carbohydrates of FMX and IIM, a heat treatment at 132°C and pH 5.4, may induce the degradation of polysaccharides. Yet, the observation that no NMR-resonances belonging to the reducing C-1 chain end units were detected in the spectra of the FMX or IIM carbohydrates indicates that degradation did not occur to a significant extent.

Characterization of the iron-carbohydrate nanoparticles

Molecular weight distribution by GFC

GFC yielded the highest M_w values for FMX (172–188 kDa, 5 lots analyzed), followed by FCM (145–155 kDa, 2 lots), IIM (63–69 kDa, 3 lots), and IS (42–44 kDa, 2 lots). In the chromatograms of all preparations, the first eluted fraction with the highest molecular weight average corresponds to the iron-carbohydrate nanoparticles (Figure 6). For FCM, IIM, and FMX, further fractions, which eluted after the first fraction and before the negative solvent peak, can be assigned to weakly-bound or dissociated carbohydrate. The position of these peaks (or shoulder, in the case of FCM) is in good agreement with the molecular weight of the respective stabilizing carbohydrates. Carboxymaltose (in FCM) and PSC (in FMX) have a higher molecular weight and therefore elute earlier than isomaltoside 1,000 in IIM. Since the FMX solution contains additional PSC as an excipient (European Medicines Agency 2012), the observed peak also includes this material. In the case of IIM, the carbohydrate peak probably also contains the citrate which is mentioned as an additional stabilizing compound by the manufacturer (Medice Pharma GmbH&Co. KG, Iserlohn, Germany 2011) and which elutes together with isomaltoside 1,000. The material eluted after the solvent peak stems from components with a significantly smaller molecular weight. For IS, two peaks are found after the solvent peak, the larger one corresponding to sucrose and chloride, and the smaller peak probably to a mixture of hydroxide and carbonate. In the FCM and the IIM chromatograms, the last peak arises from chloride which is present in the two solutions in comparable amounts. Finally, the last peak of the FMX chromatogram has been assigned to mannitol, which is a further excipient in the FMX solution [44 mg/mL, (AMAG Pharmaceuticals Inc., Waltham, MA, USA 2009)].

The relative order of the M_w values is in agreement with previously published data (Balakrishnan et al 2009; Jahn et al 2011), but the shapes of the chromatograms and the absolute values are different. For instance, a large discrepancy was noticed between the M_w values reported for FMX, i.e. 731 kDa (Balakrishnan et al 2009), 275.7 kDa (Jahn et al 2011), and 172–188 kDa (this work). This variation can be explained by the use of different standards, i.e. protein (Balakrishnan et al 2009) and dextran (Jahn et al 2011) standards instead of the pullulan standards used in the USP method (U.S. Food and Drug Administration 2012b) and in this work. Since the apparent density of the investigated compounds does not correspond to any of these standards, none of the reported values of the molecular weight represents an absolute measure, but they allow for a relative comparison between the samples' hydrodynamic volume. The different results for M_w are therefore not necessarily contradictory. Nevertheless, it is important to note that M_w values of different compounds can only be compared when measured relative to the same standard, a guidance that is not always followed (Munoz and Martin-Montanez 2012; Pai and Garba 2012; Rosner and Auerbach 2011).

Also the shapes of the reported elution profiles for the various preparations differ among publications. In an earlier report (Jahn et al 2011), the peak of the highest molecular weight component for FMX was observed close to the elution volume, as indicated by the authors. Thus, this peak as well as the shoulder observed at the same position for FCM probably are artifacts resulting from non-separation of higher molecular weight components. Furthermore, the lower molecular weight components detected in this work in the IS and IIM chromatograms (Figure 6) have not been detected in the earlier report (Jahn et al 2011). The exact reason for this discrepancy is not clear, as the full experimental details have not been reported by Jan et al, but it can most likely be explained by different experimental details, such as the choice of the column, detector and/or solvent.

Polarographic analysis

Polarographic analysis showed for all compounds the two expected transitions, i.e. Fe(III)/Fe(II) and Fe(II)/Fe(0) (Merli et al 2012; U.S. Food and Drug Administration 2012b), but the shape and the maxima of the first reduction peaks were characteristic for each preparation (Figure 7; Table S6, Supplementary data). In the range between -100 and -700 mV, FCM showed the narrowest and the most uniform peak with only a slight shoulder. For IS, the Fe(III)/Fe(II) peak in this range was much broader, whereas IIM showed a double peak with two nearly equally intense responses. In the intermediate potential range between about -700 and $-1,000$ mV there were essentially no transitions for IS, FCM, and IIM. The values of the reduction potential for IS and FCM differ to some extent from those reported earlier (Crichton et al 2008), which were recorded in a TRIS buffered solution at pH 7.4 for FCM and an acetate-buffered solution at pH 6 for IS (Vifor (International) Ltd., internal reports).

In contrast, FMX showed a completely different pattern with two well separated reduction bands in the range between -50 and $-1,000$ mV, with the more positive peak (max. around -250 mV) being less pronounced than the more negative peak (max. around -740 mV). A control measurement demonstrated that mannitol, present in rather large amounts as an excipient in FMX, does not show any reduction wave in the studied range (data not shown). The polarograms of all compounds showed very similarly positioned peaks for the Fe(II)/Fe(0) transitions in the range $-1,000$ to $-1,400$ mV. It has recently been claimed that the second peak in the polarogram of IS cannot be attributed to the Fe(II)/Fe(0) transition, but arises from adsorption processes (Mahmoudi and Kissner 2014). However, these polarographic analyses (Mahmoudi and Kissner 2014) were carried out with a different technique, a different electrode

and, importantly, at a different pH than those described in our study as well as in the current USP (Merli et al 2012). Thus, the relevance of these results for the present discussion is questionable.

The two distinct reduction bands for Fe(III)/Fe(II) in the FMX polarogram suggest at least two types of Fe(III) with a chemical environment with substantially different electronic properties. Possibly, the Fe(III) of the inner core of the polynuclear Fe(III)-oxide (maghemite) is more stable towards reduction, i.e. has a more negative reduction potential, than that on the surface. This property could be explained by the strong coordination of Fe(III) with oxide ions (O^{2-}) in the center of the core, as opposed to the coordination of Fe(III) with hydroxide and/or water at the surface of the polynuclear iron core. FCM, IIM ((Jahn et al 2011), this work), and IS have polynuclear iron(III)-oxyhydroxide cores with an akaganeite structure. The double peak present in the polarogram of IIM and IS but not in that of FCM seems therefore not likely to be caused by structural differences in the inner akaganeite core of the particles. The differences in the polarograms may arise from different types of iron-carbohydrate interactions, as well as from the different particle shapes (Jahn et al 2011), which may cause different surface properties. Overall, the polarograms illustrate that the stability and the electronic environment in the Fe(III) cores is different and distinct for each preparation. Thus, the polarogram can be regarded as a fingerprint of the iron cores' structure and carbohydrate interactions, as well as the thermodynamic stability of the cores (Toblli et al 2012).

Reductive degradation kinetics

The kinetic stability of IIM, FCM, and FMX was evaluated under acidic conditions in presence of ascorbic acid (Figure 8). The values of $k(\theta)$ at 10, 50, and 80% of degradation ($\theta = 0.1, 0.5$, and 0.8) are given in Table S7 (Supplementary data). The kinetics observed for IIM was comparable and in the same range as the values previously reported for different iron dextrans (Funk et al 2010; Geisser et al 1992). In contrast, FMX showed faster reductive degradation kinetics. All three compounds (FMX, IIM, FCM) show progressive, i.e. accelerating, kinetics with increasing rate constants (Figure 8; Table S7, Supplementary data) in agreement with the behavior expected for approximately monodisperse compounds (Erni et al 1984; Geisser et al 1992).

IV iron compounds with slow degradation kinetics ($k(\theta) = (15-50) \times 10^3 \text{ min}^{-1}$, $\theta = 0.5$) have been classified as robust (Geisser et al 1992; Geisser and Burckhardt 2011). This property was foreseen for FCM and IIM, based on previously reported kinetics of acid degradation (Jahn et al

2011). However, the faster degradation of FMX seems to be in contrast to the data on acid decomposition kinetics, which was slower for FMX than for IIM and FCM (Jahn et al 2011). Evidently, although FMX is very resistant against acid hydrolysis, it is more susceptible to reductive degradation than IIM and FCM.

For the first generation of IV iron preparations, a correlation was observed between the reductive degradation kinetics and the M_w value (Geisser et al 1992). This observation led to the classification of these compounds into four types according to their kinetic and thermodynamic stability and M_w (Geisser et al 1992). However, the correlation between M_w and degradation kinetics does not apply to FMX, which should be classified as strong and robust (Type I) according to its M_w of about 180 kDa but showed the relatively faster reductive degradation kinetics of a semi-robust and moderately strong (Type II) compound. On the other hand, the M_w of IIM (63–69 kDa) is typical for Type II compounds, whereas its reduction kinetics is that of a Type I compound. Therefore, this classification as well as the correlation between the M_w and the iron release kinetics (Geisser et al 1992) cannot be strictly applied to the newest generation of IV iron compounds.

Labile or weakly-bound iron

All IV iron preparations contain a certain percentage of iron which is not strongly bound to the core. Upon IV administration, this labile (or weakly-bound) iron is likely to be immediately transferred to transferrin and, if present in excess of transferrin's iron binding capacity, it may lead to the formation of non-transferrin-bound iron (NTBI) (Koskenkorva-Frank et al 2013).

As shown in Figure 9, the content of labile iron in the various IV iron preparations can be visualized in a qualitative way by mixing the iron solutions with white tea. Polyphenols in tea react with labile Fe(II) and Fe(III) species to form iron complexes which appear as a suspension of red-brown to black particles. Reaction of different IV iron preparations with the tea extract at equal iron concentrations and without adjustment of the pH (final range: pH 5.0–6.9) allowed for a classification of the preparations according to their content of labile iron. Due to the strong characteristic color of the various IV iron preparations, all of the mixtures have a more intense brown coloration than the blank tea sample. Very little visible additional discoloration was observed for FCM, FMX, and IIM, whereas a clearly visible discoloration and turbidity could be detected with IS and SFG (Figure 9). Thus, this simple experiment shows, although only qualitatively, the presence of higher amounts of labile iron in SFG and IS than in IIM, FMX, and

FCM, a result that is in agreement with quantitative measurements of labile iron (Figure 9 and references cited therein).

Labile iron can be mobilized from the surface of a polynuclear iron core by complex-forming agents and has been quantified with a variety of chelators such as EDTA (Jahn et al 2007), ferrozine (Jahn et al 2011; Stefansson et al 2011) or transferrin (Van Wyck et al 2004). Notably, depending on their affinity for iron, these chelators may also partly mobilize more strongly bound iron from the core and lead to artificially high values for labile iron. Therefore, the various methods used to measure labile iron often yield different absolute amounts for the same IV iron preparation (Figure 9 and references cited therein). Nevertheless, the reported amounts of labile iron are mostly in the same order of magnitude and relative differences obtained with the various methods are comparable.

In some studies, when comparing the properties of different IV iron preparations, a distinction was made between "free" and labile iron (Jahn et al 2011). Free iron was used as a synonym for dialyzable iron present in diluted iron preparations (Balakrishnan et al 2009; Jahn et al 2011) which was quantified either by ultrafiltration with a molecular weight cut-off of 30 kDa (Balakrishnan et al 2009) or by dialysis (with a tubing cut-off of 12–14 kDa) (Balakrishnan et al 2009; Jahn et al 2011). Clearly, under these experimental conditions, the measurement of free iron includes oligomeric forms with a high molecular weight up to the selected cut-off. In both cases, the relative amount of free iron for the different IV iron preparations was not in agreement with the labile iron results (Figure 9). In particular, IS showed significantly lower amounts of free iron than iron dextran (Balakrishnan et al 2009; Jahn et al 2011), a result that is difficult to rationalize with the known physico-chemical and clinical data and thus questions its validity. Notably, for all preparations the absolute amounts of free iron were very low, and thus it was concluded that free iron is not likely to induce the formation of NTBI in the blood. Importantly, the amount of free iron is included in the quantification of the labile iron (Balakrishnan et al 2009; Jahn et al 2011), which thus is a much more relevant parameter.

"Free" iron is a frequently encountered term in the medical literature which, unfortunately, is used indistinguishably as a synonym for labile (Pai and Garba 2012; Steen et al 2013; von and Adamson 2012) and dialyzable iron in IV iron preparations (Balakrishnan et al 2009; Jahn et al 2011; Van Wyck 2004), as well as for bleomycin-detectable iron (often also called catalytic iron) (Balakrishnan et al 2009; Zager et al 2004) and NTBI (Stefansson et al 2011). From a chemistry point of view, free iron is defined as $[\text{Fe}(\text{H}_2\text{O})_6]^{2+}$ or $[\text{Fe}(\text{H}_2\text{O})_6]^{3+}$, and their deprotonated forms (Balakrishnan et al 2009; Jahn et al 2011). Because of the large values of the acid dissociation

constant of water coordinated to Fe(III) ($pK_a = 2.2$) (Lippard and Berg 1994), deprotonated species are generated even at low pH values. These species rapidly dimerize, oligomerize, and lead to precipitation of the formed Fe(III)-hydroxo complexes (Crichton 2009; Lippard and Berg 1994). Noteworthy, at pH 7.0 $[Fe(H_2O)_6]^{3+}$ exists in solution only at concentrations $\leq 10^{-18}$ M (Crichton 2009), and the concentration of free iron (in its Fe(III) form) is maximally around 10^{-10} M (Cornell and Schwertmann 2003). Under physiological conditions, significant concentrations of water-soluble Fe(III)-species can only be obtained in the presence of strong chelating ligands (Crichton 2009; Lippard and Berg 1994). The reduced form $[Fe(H_2O)_6]^{2+}$ is more soluble but is rapidly (auto)oxidized under physiological conditions (Crichton 2009; Koskenkorva-Frank et al 2013). Thus, discussions about free iron are meaningless, and the analysis of free iron (as dialyzable iron) in IV iron preparations at a pH 5–11 also quantifies oligomers generated during the long dialysis procedure (up to 24 h) (Balakrishnan et al 2009; Jahn et al 2011).

In summary, the different methods, including the qualitative tea-test experiment, show similar trends of labile iron contents with $SFG \geq IS > LMWID \geq FMX \approx IIM \approx FCM$, which roughly mirrors the maximal recommended single doses of the different preparations (Table 1). Thus, if applied at the recommended doses, all IV iron preparations should lead only to a minimal, transient formation of NTBI (Koskenkorva-Frank et al 2013).

Conclusion

All IV iron preparations are colloidal solutions of compounds made of polynuclear Fe(III)-(oxyhydr)oxide cores stabilized by carbohydrates. Although the active ingredients are rather similar, and the products are used for similar indications, clinically-relevant properties such as their maximum recommended doses and serum terminal half-lives are significantly different. These dissimilarities among iron-based nano-colloidal products are not surprising because the nano-particles vary in structure and size as well as in the type and strength of bonds between the core and the stabilizing carbohydrates (Geisser et al 1992; Kästele et al 2014). The particle core as well as the carbohydrates (except for IS and SFG) are polymers, each with a specific molecular weight distribution and, therefore, the exact characterization of these compounds is accordingly difficult. This problem has recently been recognized by the regulatory authorities, and there is an ongoing discussion regarding the minimal requirements for approval of intended copies of non-biological complex drugs (Borchard et al 2012; Crommelin et al 2014; Schellekens et al 2014). For the registration of a generic form of an IV iron preparation such as IS and SFG,

the FDA requires a number of analyses in addition to those that are given in the monographs (U.S. Food and Drug Administration 2012a; U.S. Food and Drug Administration 2013). Techniques used in this work, such as polarography, XRD, and Mössbauer spectroscopy, have been mentioned by the FDA in the context of a better characterization of SFG (Woodcock 2011). The conflicting XRD results for the IS core reported in different studies apart from this work (Funk et al 2001; Jahn et al 2011; Kudasheva et al 2004) further illustrate the difficulty to accurately characterize these materials. We demonstrated that Fe K-edge XANES spectroscopy is helpful to characterize the poorly crystalline samples, a result which highlights the importance of the right choice of the applied methods. Finally, we showed that the molecular weight distribution parameters strongly depend on the standards used for the calibration of the chromatography column and do not represent absolute values. Clearly, more work is needed to elucidate the exact cellular uptake and degradation mechanisms of nano-colloidal IV iron preparations in order to determine which physico-chemical properties are fundamental for their clinical safety and efficacy.

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Conflict of interest

S. Neiser, M. Wilhelm, M. Braitsch, F. Funk, E. Philipp, and S. Burckhardt are employees of Vifor (International) Ltd.. D. Rentsch investigated the structure of the carbohydrate components by NMR, U. Dippon and A. Kappler examined the core structures by Mössbauer spectroscopy, P. Weidler investigated the core structures by XRD, and J. Göttlicher and R. Steininger examined the core structures by Fe K-edge XANES. The measurements for these studies were paid for by Vifor (International) Ltd..

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 907
 908
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910

911 **Tables**

912

913 **Table 1 Chemical classification, dosing, and terminal half-life of the most important**
 914 **current IV iron preparations**

Brand names	Ferlecit [®]	CosmoFer [®] , INFeD [®]	Venofer [®]	Ferinject [®] , Injectafer [®]	MonoFer [®]	Feraheme [®] , Rienso [®]
Active ingredient	Sodium ferric gluconate (SFG)	Low molecular weight iron dextran (LMWID)	Iron sucrose (IS)	Ferric carboxymaltose (FCM)	Iron isomaltoside 1,000 ^a (IIM)	Ferumoxytol (FMX)
Carbohydrate	Gluconate	Dextran	Sucrose	Carboxymaltose	Isomaltoside 1,000 ^a	PSC ^b
Max. single Fe dose (min. admin. time) ^c	125 mg (10–60 min)	20 mg/kg b.w. (4–6 h)	200 mg (10–30 min)	1,000 mg (15 min)	20 mg/kg b.w. (60 min)	510 mg (17 s)
Terminal half-life (dose in mg Fe) ^d	1.42 h (125)	27–30 h (500–2,000)	5.3 h (100)	7.4/9.4 h (100/1,000)	20.8/22.5 h (100/200) ^e	14.7 h (316)

915 ^aIn German SPC (Medice Pharma GmbH&Co. KG, Iserlohn, Germany 2011): Iron citrate isomalto-oligosaccharide
 916 alcohol-hydrate complex

917 ^bPSC, polyglucose sorbitol carboxymethylether

918 ^cMost common maximal dose and corresponding minimal administration time: the exact posology may vary between
 919 markets. Please refer to local prescribing information.

920 ^d (Geisser and Burckhardt 2011) and references cited therein.

921 ^e (Nordfeld et al 2012).

922 b.w., body weight.

924 **Table 2 Overview of the results from XRD, XANES spectroscopy, and Mössbauer**
925 **spectroscopy**

926 ¹ n/a (not applicable): akaganeite most likely but no clear identification of the core mineral by XRD, see
927 text.
928 ² not determined.

930

931 **Table 3** Labile or “free” iron content in IV iron preparations assessed by different analytical methods.

	Method	unit	SFG	IS	LMWID	FCM	FMX	IIM	Ref.
Labile, weakly-bound iron	EDTA <i>in vitro</i> - 1 min	[%]	2.1	1.1	0.4	n/a	n/a	n/a	(Funk 2010)
	- 19.3 h	[%]	4	4	1				(Funk 2010)
	Ferrozine <i>in vitro</i> (human serum)	[%]	3.2	3.5	2	0.5	1	1	(Jahn 2011)
		[%]		5.6	1.6				(Jahn 2007)
	Ferrozine <i>in vivo</i> (humans)	[μM]	n/a	27.9	7.4	n/a	n/a	n/a	(Jahn 2007)
	Transferrin <i>in vitro</i>	[%]	5.8	4.5	3.4	n/a	n/a	n/a	(Van Wyck 2004)
BDI	Bleomycin - rat serum	[%]	1.4	0.69	0.19	n/a	0.07	n/a	(Cornell 2003)
	- human serum	[%]	0.96	0.89	0.40		0.15		(Cornell 2003)
	- rat <i>in vivo</i>	[mM]	1.69	0.92	0.65		0.33		(Cornell 2003)
Dialyzable/ filterable iron	Ultrafilterable iron (no pH adjustment, 30 kDa)	[%]	2.36	0.038	0.298	n/a	0.001	n/a	(Cornell 2003)
	Dialysis (MWCO 12–14 kDa) - pH 7	[%]	1.338	0.067	0.207	<0.002	<0.002	<0.002	(Jahn 2011)
	- no pH adjustment	[%]	n/a	n/a	0.172	0.262	0.005	0.014	(Jahn 2011)
	Hemodialyzer (PO ₄ ³⁻ -buffer)	[%]	5	3	<1.0	n/a	<1.0	n/a	(Cornell 2003)

BDI, Bleomycin-detectable iron; FCM, ferric carboxymaltose; FMX, ferumoxytol; IIM, iron isomaltoside 1,000; IS, iron sucrose; LMWID, low molecular weight iron dextran; MWCO, molecular weight cut-off; n/a, not available; NTBI, non-transferrin-bound iron; Ref., reference; SFG, sodium ferric gluconate

932

933

934 **Figure captions**

935 **Fig 1** Mössbauer spectra of FMX, IIM and FCM recorded at 77 K (left) and 5 K (right).
936 Colored dots represent the Mössbauer signal, dark grey lines the fit, and light gray
937 lines the single models used for fitting of the spectra. Modeling parameters are given
938 in Table S2 (Supplementary data)

939

940 **Fig 2** X-ray diffractograms of FMX (a), IS (b), FCM (c), and IIM (d). The diffractograms
941 show the raw data (grey dots), fitted model (red), sub-patterns of the model phases
942 (blue and green), and in the FMX spectrum (a) also the internal standard corundum
943 (light blue). The model phases are maghemite for FMX (a), goethite (blue) and
944 akaganeite (green) for IS (b), and akaganeite for FCM and IIM (c, d). Below the
945 diffractograms, the difference curves are shown (gray) and the peak positions of the
946 indicated phases are marked by vertical lines

947

948 **Fig 3** Fe K X-ray absorption near edge structure (XANES) spectra of IS and IIM compared
949 to reference spectra of akaganeite, goethite, lepidocrocite, 2L- and 6L-ferrihydrite
950 (2L-Fh, 6L-Fh): Overview of the entire XANES energy range (a), details from the pre-
951 edge range (b), from the main peak (c), and from the post-edge and beginning
952 EXAFS range (d). The legend in (a) applies also to (b, c and d)

953

954 **Fig 4** Different chemical species identified in the carbohydrate components of FMX and IIM
955 and in the dextran reference materials. R: glycoside polymer

956

957 **Fig 5** Regions of interest of 1D ^1H NMR spectra of the carbohydrate components of IIM
958 and FMX and of the different reference dextrans with assignments to chemical
959 species shown in Figure 4. Characteristic spectral regions and number of protons
960 contributing to the signal intensities: 5.33 ppm (1,3-br., 1 H), 5.24 ppm ($\text{C}\alpha$, 1 H),
961 5.18 ppm (D, 1 H), 4.98 ppm (A, B, Cred', E and F, 1 H each), 4.67 ppm ($\text{C}\beta$, 1 H),
962 4.00–4.25 ppm (D, E and F, 2 H each), 3.45 ppm (B and D, 1 H each), 3.26 ppm ($\text{C}\beta$,
963 1 H)

964

965 **Fig 6** Gel filtration chromatography of IS (Venofer[®] lot 091201), FCM (Ferinject[®] lot
966 169001), FMX (Feraheme[®] lot A56996A), and IIM (MonoFer[®] lot 042838-3)

967

968 **Fig 7** Polarograms of FMX (Feraheme[®] lot 1061002), IIM (MonoFer[®] lot 042838-3), IS
969 (Venofer[®] lot 091201), and FCM (Ferinject[®] lot 169001). Potentials are given vs.
970 SHE; the reduction potentials are given in Table S6, Supplementary data

971

972 **Fig 8** Reductive degradation kinetics shown as the extent of decomposition (θ) of IIM,
973 FCM, and FMX as a function of time. The two curves for IIM are nearly congruent.
974 [Calculated according to Erni (Erni et al 1984)]

975

976 **Fig 9** Visualization of labile iron content by mixing iron preparations (0.1 mg Fe/mL) with
977 white tea. Labile or “free” iron reacts with polyphenols in tea to form a suspension of
978 red-brown to black iron complex particles.

Figure 1

Figure 2

Figure 3

Figure 4

Reducing chain ends (Dextran):

Branches (PSC):

Carboxymethylated species (PSC):

Chain glycosides and reduced chain ends (Dextran, PSC):

Figure 5

Figure 6

Figure 7
-80

Figure 8

Figure 9

1 **Supplementary data:**
2 **"Physico-chemical properties of the new generation IV iron preparations**
3 **ferumoxytol, iron isomaltoside 1'000, and ferric carboxymaltose", *Neiser et. al.***

4
5 **1. X-ray diffraction (XRD), Mössbauer spectroscopy, and X-ray absorption near edge**
6 **structure (XANES) spectroscopy**

7 **Table S1 Overview of the XRD experimental parameters**

Sample	Lot no.	Instrument	Radiation	Scan range (°2θ)	Step / time (°2θ / sec)	Detector
Feraheme®	09060402	Bruker D8	Co Kα _{1,2}	8 – 72	0.025 / 8	Scintillator
Venofer®	947100	Bruker D5000	Cu Kα _{1,2}	5 – 75	0.030 / 20	Scintillator
MonoFer®	949171-1	Bruker D8	Cu Kα _{1,2}	4 – 84	0.024 / 120	Lynxeye® PSD
Ferinject®	267100M	Bruker D5000 rotating anode	Mo Kα _{1,2}	2 – 40	0.020 / 20	Scintillator

8

9

Table S2 Blocking temperature and minerals identified by Mössbauer spectroscopy and modeling parameters

Compound	Results		Modeling parameters					
	Blocking T (K)	Mineral suggested	T (K)	Model	CS (mm/s)	QS (mm/s)	H (T)	Area (%)
FMX (Feraheme [®])	73	maghemite	77	doublet	0.49	0.50	–	65
				sextet	0.46	0	46.4	35
			5	sextet 1	0.34	0	51.5	54
				sextet 2	0.56	–0.04	51.9	46
IIM (MonoFer [®])	56	akaganeite	77	doublet	0.47	0.79	–	66
				sextet	0.50	0	43.5	34
			5	sextet	0.48	–0.06	47.6	100
FCM (Ferinject [®])	114	akaganeite	77	doublet	0.44	0.80	–	34
				sextet 1	0.47	–0.18	42.7	28
				sextet 2	0.43	–0.24	26.8	38
			5	sextet 1	0.59	–0.12	46.7	57
				sextet 2	0.36	0.06	47.2	43

CS: center shift, describes the energy shift of the spectrum relative to α -Fe at room temperature, QS: quadrupole splitting, H: magnetic hyper fine field

15

16 **Table S3 Overview of the results from XRD**

Sample	XRD							
	Core mineral	Space group	Lattice constants				GOF	Domain size (nm)
			a_0 (nm)	b_0 (nm)	c_0 (nm)	β (°)		
FMX	maghemite (γ -Fe ₂ O ₃)	#96 P4 ₃ 2 ₁ 2	0.839	n/a	2.502	n/a	6.8	10
FCM	akaganeite (β -FeOOH)	#12 I2/m	1.069	0.302	1.034	92.4	1.4	4-5
IIM	akaganeite (β -FeOOH)	#12 I2/m	0.998	0.300	1.035	91.2	16.6	3
IS	n/a ¹	n/a ¹	n/a ¹	n/a ¹	n/a ¹	n/a ¹	n/a ¹	n/a ¹

17 ¹ not applicable; akaganeite most likely but no clear identification of the core mineral by
18 XRD, see main text. Reference value of the lattice constants from the literature:
19 maghemite: $a_0 = 0.833$ nm and $c_0 = 2.499$ nm [reference 56, main text]; akaganeite:
20 $a_0 = 1.0600$ nm, $b_0 = 0.3034$ nm, $c_0 = 1.0513$ nm and $\beta = 90.24^\circ$ [reference 57, main text]

21

22 Due to the small particle sizes, an assignment of the mineral structure and a calculation of the
23 lattice constants was not possible by XRD for IS, and for IIM the incertitude is reflected in the
24 high GOF value. For these preparations, XANES spectroscopy was performed for a
25 determination of the short range structure.

26

27

28 **2. Linear Combination Fits of Fe K XANES spectra of IS and IIM**

29 Combinatorial Linear Combination Fits (LCF) have been performed for the Fe *K*-edge XANES
30 spectra of IS (Venofer[®]) and IIM (MonoFer[®]) with five reference spectra: akaganeite, goethite,
31 lepidocrocite, 2L-ferrihydrite (2L-Fh) and 6L-ferrihydrite (6L-Fh) (reference [43], main text). Very
32 small fractions around and below 1% are likely to be mathematical effects and were thus not
33 considered as relevant.

34 The best fit for IS spectrum resulted in 73.1% akaganeite, 12.0% and 15.2% 2L-Fh and 6L-Fh
35 fraction, respectively (Fig. S1A), without normalization of the fractions to 100%. Still, a pure
36 akaganeite fraction yielded also a very reasonable result (Fig. S1B).

37 The best fit for the IIM spectrum was obtained for akaganeite (84.2%) and 6L-ferrihydrite
38 (16.6%) fraction (not normalized to 100%, Fig. S2A). Again, a pure akaganeite fraction also gave
39 a very reasonable result (Fig. S2B).

40 The fitted weights, normalized to 100%, and the R-factors are summarized in Table S4. The
41 normalized values deviated little from the un-constrained sums cited above (max. 0.8%). For
42 ferrihydrite, the sum of 2L- and 6L-ferrihydrite is given because spectra are similar and separate
43 designation is not justified.

44 Because of the small differences in R-factors between the fits that resulted in akaganeite plus
45 ferrihydrite and the fits with akaganeite only, the IS and IIM spectra may also be described
46 satisfactorily by akaganeite only out of the pool of the five reference spectra that have been
47 used here. This conclusion is supported by the fact that additional components do not give a
48 better fit for certain XANES features like the top of the main peak and the pronounced shoulder
49 at the high energy flank of the main peak (Figs. S1A, B, S2A, B).

50 For the precision of the LC-fit of XANES spectra, values of about $\pm 10\%$ seem to be reasonable.
51 The fits have been performed as combinatorial fits where no combinations have been excluded,
52 resulting in 26 fits per sample when using five reference spectra. Because the energy was
53 calibrated more precise than 0.1 eV for all spectra, no energy shift has been allowed during the
54 fits. The only constraints were that weights have to be between 0 and 1 (100%). Because of the
55 relatively high error, decimal places should not be given when using these values elsewhere.

Figure S1a Best convergence of the combinatorial linear combination fit of the Fe K-edge XANES spectrum of IS with akaganeite (73.1%), 2L- and 6L-ferrihydrite (12.0% and 15.2%, respectively) spectral fractions

Figure S1b Good convergence of combinatorial linear combination fit of the Fe K-edge XANES spectrum of IS with an akaganeite fraction only (100%); a second component (goethite here) has been fitted to zero

Figure S2a Best convergence of the combinatorial linear combination fit of the Fe K-edge XANES spectrum of IIM with akaganeite (84.2%) and 6L-ferrihydrite (16.6%)

Figure S2b Good convergence of the combinatorial linear combination fit of the Fe K-edge XANES spectrum of IIM with an akaganeite fraction only (100%); a second component (goethite here) has been fitted to zero

Table S4 Summary of the results of the main spectral fractions from linear combination fits normalized to 100% for the IS and IIM Fe K-edge XANES spectra with references spectra of akaganeite (Akag), goethite, lepidocrocite, 2L- and 6L-ferrihydrite (2L-Fh, 6L-Fh). 2L- and 6L-ferrihydrites are summed to a general ferrihydrite (sum Fh) component because the spectra are similar

Fractions	Akag (%)	Sum Fh (%)	R-factor x 10 ⁻⁵
IS (Venofer [®]), fit results 1	72.9	27.1	6.2
IS (Venofer [®]), fit results 2	100.0	0.0	21.7
IIM (MonoFer [®]), fit results 1	83.5	16.5	6.6
IIM (MonoFer [®]), fit results 2	100.0	0.0	14.5

3. Characterization of the carbohydrate components of IIM and FMX by ^{13}C NMR

Figure S3 Regions of interest of 1D ^{13}C NMR spectra of the IIM and FMX carbohydrates and of the different reference dextrans (chemical structures in Figure 4, main text) with assignments of characteristic spectral regions and number of carbon atoms contributing to the signal intensities: 100.2 ppm (1,3-br., 1 C), 98.4–99.2 ppm (A, B, Cred', E and F, 1 C each), 97.2 & 77.0 ppm ($\text{C}\beta$, 1 C each), 96.7 ppm (D, 1 C), 93.3 ppm ($\text{C}\alpha$, 1 C), 84.6 ppm (E, 1 C), 80.1–80.7 (D and F, 1 C each), 67.2 ppm (F, 1 C), 63.4 ppm (Cred, 1 C), 61.4 ppm (B and 1,3-br., 1 C each). The inserts in the spectra of reduced and carboxymethylated Dextran 1'000 and the FMX carbohydrate show the carboxyl region with 1 C each of species E, F and D at 179.8, 179.0 and 178.7 ppm, respectively

95 **Table S5** Chain lengths (CL), carboxymethylation substitution degrees (SDCM) and
96 number average molecular weight (M_n) determined from ^1H and ^{13}C NMR data
97 for the carbohydrate components isolated from FMX and IIM, and dextran
98 reference samples

Sample	Lot	Data from ^1H NMR			Data from ^{13}C NMR		
		CL	SD _{CM}	M_n (g/mol)	CL	SD _{CM}	M_n (g/mol)
Dextran 1'000	BCBD4347V-02-002	6.9	0	1'133	6.2	0	1'029
Dextran 3–4'000	303820	16.7	0	2'721	18.6	0	3'040
Isomaltoside 1'000 (IIM)	949171-1	4.7	0	782	4.7	0	777
Reduced Dextran 3–4'000	303820	17.7	0	2'886	18.5	0	3'025
Carboxymethylated Dextran 1'000	BCBD4347V-02-002	8.4	0.19	1'477	9.1	0.20	1'598
PSC (FMX)	09060402	19.5	0.20	3'415	21.4	0.22	3'758
	10012802	21.7	0.23	3'828	21.4	0.18	3'702

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4. Polarographic analysis of iron-carbohydrate nanoparticles

Table S6 Reduction potentials vs. SHE obtained from the polarograms of IS, FCM, FMX, and IIM

Compound		Reduction potential peak maxima response vs. SHE (mV)		
		Peak 1	Peak 2	Peak 3
FMX	lot 1061002 ¹	−229	−744	−1'168
	lot A56996A	−252	−747	−1'182
	lot 09060402 ²	−255	−782	not specified
IIM	lot 042838-3 ¹	−347	−501	−1'196
	lot 951208-1	−329	−514	−1'189
FCM	lot 169001 ^{1,3}	−333	n/a	−1'231
IS	lot 091201 ^{1,3}	−494	n/a	−1'151

¹Polarograms of these lots are depicted in Figure 7, main text

² (Toblli et al 2011)

5. Reductive degradation kinetics of iron-carbohydrate nanoparticles

Table S7 Kinetics data on reductive degradation of different IV iron preparations

Iron preparation	Lot no.	k(θ) x 10 ³ (min ⁻¹)			<i>M_w</i> (kDa)
		$\theta = 0.1$	$\theta = 0.5$	$\theta = 0.8$	
Iron dextran BP/USP ¹	015109	20	34	67 ($\theta = 0.9$)	103
Iron dextran 5 ¹	911308	23	34	66 ($\theta = 0.9$)	523
Iron dextran 20 ¹	985108	12	27	50 ($\theta = 0.9$)	445
IIM	951208-1	21	41	62	69
	042838-3	21	41	64	63
FCM	94300022	17	33	52	155
	169001	18	36	57	145
FMX	09060402	40	71	108	185 ²
	A56996A	31	60	84	188
IS ¹	10061002	38	68	102	—
	750208	107	89	117 ($\theta = 0.9$)	—

¹ (Geisser et al 1992); ² (Toblli et al 2011)

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112 **References**

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