Ferrozine Assay for Simple and Cheap Iron Analysis of Silica-Coated Iron Oxide Nanoparticles

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ABSTRACT: The Ferrozine assay is applied as an accurate and rapid method to quantify the iron content of iron oxide nanoparticles (IONPs) and can be used in biological matrices. The addition of ascorbic acid accelerates the digestion process and can penetrate an IONP core within a mesoporous or solid silica shell. This new digestion protocol avoids the need for hydrofluoric acid to digest the surrounding silica shell and provides an accessible alternative to inductively coupled plasma methods. With the updated digestion protocol, the quantitative range of the Ferrozine assay is 1 – 14 ppm.

INTRODUCTION:

The Ferrozine assay is a simple and accurate colorimetry assay for iron quantification. The chelator, 3-(2-pyridyl)-5,6-bis(4-pheylsulfonic acid)-1,2,4-triazine (Ferrozine), absorbs at 562 nm when bound to ferrous iron, allowing for quantification [1, 2]. The original protocol by Carter *et. al.* was developed for the quantification of endogenous iron in blood[3]. Since its inception, the Ferrozine assay has been applied to the detection of iron within a variety of biological and geological samples [1, 4-9].

Inductively coupled plasma (ICP) methods are considered the gold standard for iron quantification and have a lower limit which ranges from ppb – ppt based on the detection method [10, 11]. However, several challenges are associated with using ICP methods, such as the costly equipment, necessary weekly maintenance, special training, and great care to remove any impurities since ICP is very sensitive to interferences caused by polyatomic and environmental iron contamination [12]. Therefore, the Ferrozine assay can provide a cheaper and more accessible iron quantification method.

Iron-oxide nanoparticles (IONPs) are receiving increased attention based on their potential for biomedical applications such as MRI contrast, hyperthermia therapy, regenerative medicine, cell targeting, and drug delivery [13-19]. Quantification of iron content is vital for accurate IONP characterization and IONP interaction within *in situ* systems. Ferrozine assay procedures have been applied to IONPs within samples containing biological cells [20-24]. However, in performing a comparison between Ferrozine assay, Ferene assay, and ICP-OES, Hedayati *et. al.* commented on the importance of full IONP digestion to ensure accurate iron quantification [24].

Silica-based coatings are a promising candidate to enhance IONP stability against aggregation and degradation when deployed within biological solutions [25, 26]. The synthesis of the silica shell can be easily tailored to adjust physical parameters, such as shell thickness, surface functionality, and porosity [27, 28]. However, silica-based coatings provide an additional challenge for iron quantification, since hydrofluoric acid is necessary to dissolve the silica shell. With the Ferrozine assay in particular, the presence of silica has been documented to effect the iron quantification due to the potential to hinder full digestion of the IONP [29]. Herein, we demonstrate a digestion method which fully dissolves an IONP encased in a mesoporous or solid silica shell and the impact this digestion has on the Ferrozine assay.

EXPERIMENTAL SECTION:

Materials and Reagents:

Ammonium acetate (631-51-8) was purchased from Mallinckrodt Ltd, United Kingdom. Ascorbic acid (50-81-7) was purchased from Acros Organics, USA. Mohr's salt (7783-85-9) was acquired from Macron, USA. Concentrated hydrochloric acid (7647-01-0), Ferrozine (63451-29-6), neocuproine (484-11-7), and polyvinylpyrrolidone (PVP-10, average molecular weight 10,000) were purchased from Sigma Aldrich, USA. Chlorotrimethylsilane (TMS, >99%) was purchased form Fluka, USA. 2-[methoxy(polyethyleneoxy)-propyl]9-12-trimethoxysilane (PEG-silane, molecular weight 596-725 g/mol, 9-12 EO) was obtained from Gelest, Inc., USA. All reagents were used as received. All glassware used during this procedure was acid washed with concentrated hydrochloric acid.

Iron Oxide Nanoparticles:

This experiment compared the same IONP core without a shell, with a mesoporous silica shell (msIONP), and with a solid silica shell (ssIONP). The IONP core was purchased from Ferrotec (EMG-308; Bedford, NH, USA). The msIONPs were synthesized as described previously [30]. The shell thickness was measured using transmission electron microscopy (TEM) to be 15 ± 3 nm. The ssIONPs were synthesized following a modified Stober synthesis[31]. The stability of the IONPs during synthesis was maintained using PVP-10 at the same ratio used for the msIONP synthesis (2:3 IONP:PVP-10) [30]. After the solid silica shell was condensed around the IONP core, PEG-silane and TMS were added. The TEOS:PEG-silane:TMS ratio was kept at 80:20:3. The shell thickness was measured to be 17 ± 2 nm by TEM. The msIONPs and ssIONPs were stored after lyophilization. The msIONPs and ssIONPs were dispersed in water. All stock solutions were sonicated for 10 minutes before use and diluted to approximately 230 ppm for iron concentration validation.

Silica shell thickness was verified using transmission electron microscopy (TEM). TEM was performed on a Tecnai T12 transmission electron microscope operating at 120 kV. Nanoparticle samples were deposited on a 200 mesh copper TEM grid with carbon and Formvar supports (Ted Pella, Inc) by briefly dipping the grid into an ethanol or aqueous suspension of silica-coated IONPs and allowing the grid to air-dry.

Sample Digestion:

Samples were digested at 600 µL volumes in a block heater set to 60°C. Literature precedent IONP digestion methods for a Ferrozine assay varied in acid and concentration. In particular, hydrochloric acid ranged from 0.3 to 12 M [20-23]. Kalambur *et. al.* deviated from the use of only hydrochloric acid for digestion to a combination of both hydrochloric and ascorbic acid [22]. We selected 0.3 M concentrations to return to the acid concentration recommendation from Fish *et. al.* [1]. A comparison was made between samples digested in 0.3 M hydrochloric and 0.3 M ascorbic acids or only 0.3 M hydrochloric acid. Digestion times were varied between 3 and 24 h. Samples were assessed every 30 minutes during digestion to check if the IONPs had crashed out of solution. If the IONPs had crashed out of solution, the samples were agitated by vortexing for 5 s. Each digested sample was split into two portions, one for analysis by Ferrozine assay and the other for ICP-OES.

Colorimetric Assay:

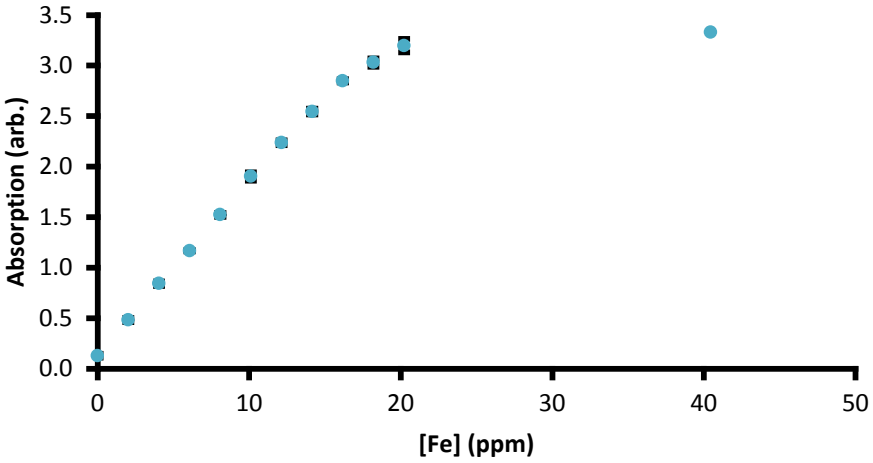

Figure 1: An iron(II) standard calibration for Ferrozine assay using Mohr's salt. The measurements range from 0 to 40 ppm demonstrating the limit of the linear range (1 – 14 ppm). Error bars demonstrate the standard deviation of the absorption measurements (n = 3).

A detailed description of both the sample digestion and colorimetric assay is given in the supplemental information. The iron-chelating reagent was prepared by dissolving ammonium acetate, ascorbic acid, Ferrozine, and neocuproine in water to have final concentrations of 5 M, 2 M, 6.5 mM, and 13.1 mM, respectively. Each assay was prepared within a 96-well plate. Each individual well had a ratio of 2:1 sample:iron-chelating reagent. The assay was allowed to incubate at room temperature in the dark for 0.5, 1, 2, and 24 h. The absorption was measured at 570 nm using a BioRad Microplate Reader, iMark (Hercules, CA, USA). An internal calibration was prepared on the 96-well plate using an iron (II) stock solution prepared from Mohr's salt. The iron (II) stock solution was prepared on the date of use and was prepared at concentrations

of 0 to 40 ppm. The digested IONP samples were diluted to approximate concentrations ranging from 0.5 to 20 ppm for further analysis. As shown in Figure 1, with our experimental setup, we observed the linear range to be between 1 – 14 ppm. This assay has been demonstrated to quantify iron concentrations as low as 42 ppb by increasing the development time of the assay [32]. The results of the assay were compared to iron concentrations measured using inductively coupled plasma-optimal emission spectroscopy (ICP-OES).

Iron Concentration Validation:

ICP-OES of the iron concentration was performed on a Thermo Scientific iCAP 6500 dual-view ICP-OES (West Palm Beach, FL, USA) with 1150W power. To fully dissolve the silica shell, a 5% by volume concentration of hydrofluoric acid was obtained by adding trace metal grade concentration hydrofluoric acid to the digested samples. The samples were subsequently diluted to an approximate iron concentration of 1.5 ppm and 2% nitric acid by volume, using trace metal grade concentrated nitric acid. Calibration standards were prepared using single-element standard solutions (SPEX CertiPrep) in 2% nitric acid with an yttrium internal standard.

Bland-Altman Assessment:

The iron concentrations measured by ICP-OES and in the linear range (1 – 14 ppm, see Figure 1) of the Ferrozine assay were compared using a ratio Bland-Altman assessment [33]. If the methods perfectly matched, the bias should be 1. A positive bias indicates the Ferrozine assay

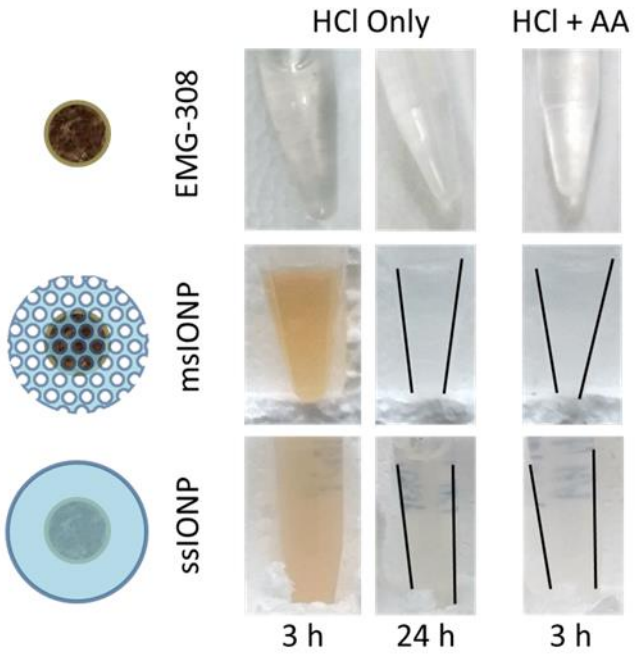

Figure 2: Digestion solution color change. Digested IONPs appear clear in color, while undigested IONPs have a brown hue. IONP cores (EMG-308) all become a clear color under all digestion solutions and time points. IONPs with a silica shell (msIONPs & ssIONPs) maintained an obvious brown hue when digested in hydrochloric acid for 3 h. Although difficult to distinguish within the image, the 24 h hydrochloric acid digestion of ssIONP still maintained a subtle brown hue. A black outline was added to images of samples with difficult to distinguish edges.

measures a lower iron concentration compared to ICP-OES. Statistical analysis was performed using GraphPad Prism 7.01.

RESULTS & DISCUSSION:

The change in solution color was an indication of the progress of digestion. During digestion, samples were observed to change from a brown opaque solution to a transparent clear (Figure 2). The presence of the silica shell was observed to have a visual impact on the speed of IONP digestion. Both msIONPs and ssIONPs maintained a brown hue when digested in only hydrochloric acid for 3 h. Furthermore, the ssIONPs were not fully clear after 24 h in hydrochloric acid. All three nanoparticles visually appeared to be fully digested after three hours in the hydrochloric and ascorbic acid mixture. The observation of a clear solution under the same conditions but at 24 h indicates that the presence of the silica shell delays the digestion process. Although solutions appeared clear earlier (for some solutions as quick as 30 min), the 3 h time point was used to compare directly with the digestion efficacy of only hydrochloric acid.

Assessment of the digested IONP solutions with the Ferrozine assay reinforced most of the observations based on solution color. The iron concentrations measured by ICP-OES and the Ferrozine assay were compared using a ratio Bland-Altman analysis (Figure 3). If there is no difference between the two methods of analysis, then the bias between the methods should be 1. Samples which were visually observed to be clear after digestion, were measured to have a bias of 1.0 ± 0.1 when the assay was allowed to develop for 24 h. All other samples were determined to have a bias > 1.15, indicating that the Ferrozine assay underestimated the amount of iron

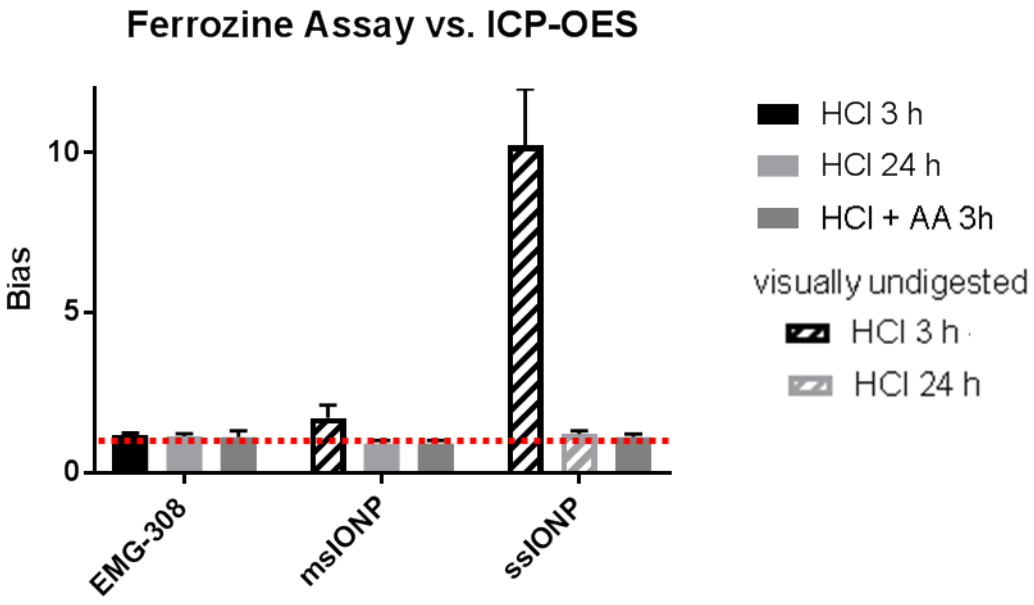

Figure 3: Acid digestion impact on Ferrozine assay with 24 h development time compared with ICP-OES. The bias calculated with a Bland-Altman comparison between the Ferrozine assay and ICP-OES iron quantification follows the same trend demonstrated in Figure 1. Samples marked with diagonal strips indicate samples that visually were not fully digested. A bias of 1 indicates no difference between the two methods that are compared (red dashed line).

present per sample. This observation matches the variation between ICP-MS and the Ferene spectroscopic assay used by Hedayati *et. al.*[24]. When the assay was developed for shorter time durations (30 min, 1 h, and 2 h), the solution color change was not always a reliable indication of complete digestion.

Samples which still had an obvious brown hue were observed to be more heavily impacted by the development of the Ferrozine assay (Figure 4, HCl 3h). However, several samples which appeared clear in color or had a weak brown hue (ssIONP in HCl 24 h) after digestion demonstrated minor changes in bias from development time (Figure S1). This indicates the presence of ascorbic acid in the chelating reagent requires additional time to fully react and allow complete conversion of Fe(III) to Fe(II). The protocol described by Fish *et. al.* indicated that the sample with the chelating solution was stable for at least 20 h; we observed stability out to 24 h [1]. After 24 h of development, the hydrochloric acid 3 h digested sample still has a bias greater than 1, implying that full digestion of the IONPs had not occurred.

Ascorbic acid was observed to accelerate the digestion of IONPs and is considered a vital component within the chelating reagent to fully reduce Fe(III) to Fe(II) [1, 32]. Previous uses of ascorbic acid during the digestion of IONPs within cells has not been explained [22]. The essential role of ascorbic acid to reduce iron for dietary uptake has been well-studied [34]. Furthermore, the kinetics and mechanisms for the redox reaction between ascorbic acid and

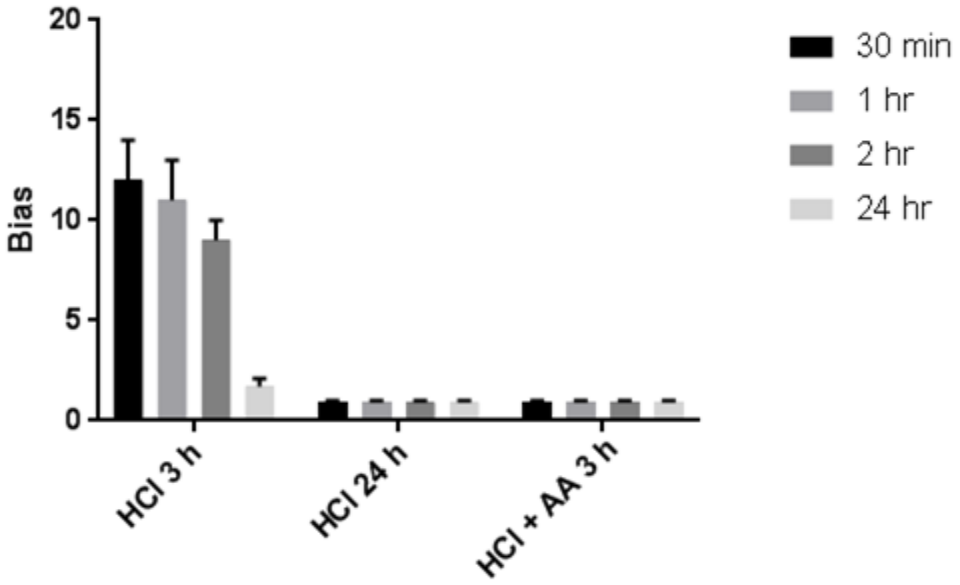

Figure 4: Development time impact on msIONP dissolution. The accuracy of the Ferrozine assay for msIONPs digested in hydrochloric acid for 3 h is increased with longer development time with the chelating reagent. However, at 24 h the values measured were still inaccurate. Whereas measurements of msIONPs digested in hydrochloric acid for 24 h and a combination of hydrochloric and ascorbic acids (AA) for 3 h are consistent regardless of development time.

iron(III)/(II) are well understood [35-37]. It is reasonable to speculate that the additional mechanisms presented by ascorbic acid allow for faster degradation of the IONP surface. However, further studies are needed to measure the kinetics and demonstrate this interaction between ascorbic acid and the IONP surface.

The digestion of the IONP core indicates that acid was able to pass through both the mesoporous and solid silica shell. The hydrothermal treatment, used with the msIONPs investigated herein, has been previously observed to inhibit the degradation of IONPs by 1 M hydrochloric acid [38]. It is more surprising that digestion of the IONP core was possible through a solid silica shell. However, Pinho *et. al.* explored the impact of solid silica shell thickness on magnetic resonance relaxivity, demonstrating water diffusion through the solid silica shell at shell thicknesses less than 30 nm [28]. Furthermore, solid silica shells have been demonstrated to have an ultramicroporous or microporous structure dependent on the washing procedures used after synthesis [39]. Therefore, it is reasonable that our solid silica shell, which was 17 nm thick, would allow passage of acid through the shell. It should be anticipated that a thicker solid silica shell would necessitate a longer digestion time.

As IONPs are more heavily investigated for biomedical applications, it is becoming more common to use the Ferrozine assay to validate doses and performance [20-23, 40]. The original Ferrozine assay protocols were developed as an alternative to ICP-OES for endogenous sources of iron within the body. These protocols were optimized to remove effects from interference agents that are common within biological specimens, such as copper [1]. When developing a Ferrozine assay protocol for a new IONP, it is important to realize there can be potential interference caused by degraded products of the IONP surface coatings. Specific concerns would be degraded components that could form oxalates, cyanide, and nitrites [41]. Additionally, as observed in this work and by others [24], full digestion of the IONP is essential for accurate iron quantification. Therefore, verification of this assay when it is initially applied to a new IONP formulation is recommended.

CONCLUSIONS:

Iron quantification with the Ferrozine assay has been verified for IONPs and demonstrated for IONPs with solid and mesoporous silica shells. The presence of ascorbic acid during the digestion step was observed to accelerate digestion. The inclusion of ascorbic acid during the digestion step of the assay was essential to achieve full digestion of the IONP in the presence of a silica shell and avoided the need to use hydrofluoric acid to fully digest the silica shell. Additionally, for the investigated IONPs, no interference between the surfactants on the IONP and the Ferrozine assay were observed.

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