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Colloidal stability and chemical reactivity of complex colloids containing Fe³⁺



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ABSTRACT

The reactivity of iron contained within insoluble colloidal metal-pyrophosphate salts was determined and compared to the reactivity of a soluble iron salt (FeCl₃). As a model system for the reactivity of iron in food products, the formation of an iron–polyphenol complex was followed with spectrophotometry. Three types of systems were prepared and their colloidal stability and reactivity studied: Fe^{3+} pyrophosphate, protein–coated Fe^{3+} pyrophosphate and mixed–metal pyrophosphates containing Fe^{3+} and a second cation M. The additional cation used was either monovalent (sodium) or divalent (M^{2+}). It was found that: (i) incorporating iron in a colloidal salt reduced its reactivity compared to free Fe^{3+} ions; (ii) coating the particles with a layer of hydrophobic protein (zein) increased stability and further decreased the reactivity. Finally, the most surprising result was that (iii) a mixed system containing more Fe^{3+} than M actually increased the reactivity of the contained iron, while the reverse, a system containing excess M, inhibited the reactivity completely.

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1. Introduction

The fortification of food products with colloidal nanoscale particles is an important field of research in the food industry, as the addition of such particles can be an efficient, simple and cost-effective way to fight mineral deficiencies both in developed and third world countries (Acosta, 2009; Velikov & Pelan, 2008). Of the essential minerals, iron is the most problematic to add to foodstuffs, mainly due to the reactivity of 'free' iron ions (from, for instance, iron sulphate) with various components of the products such as the polyphenols that are abundant in plant-based foodstuffs (Mellican, Li, Mehansho, & Nielsen, 2003). Polyphenols strongly chelate cations and the complexes with iron have intense and persistent colours (Hider, Liu, & Khodr, 2001; Mellican et al., 2003; Van Acker et al., 1996), as illustrated by the fact that gallotannic acid (a polyphenol from gallnuts) combined with Fe²⁺ has been used abundantly as a black ink for about 2000 years (De Feber, Havermans, & Defize, 2000).

In this work, various systems of iron-containing nanoscale particles were prepared, with the intention of reducing the reactivity

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of this iron, with respect to the free iron ions in solution. Next to edibility, an important prerequisite for these particles is that they should be insoluble in the food product, but they should also dissolve once consumed in order to allow the iron to be absorbed by the body. Therefore, metal pyrophosphate salts were used which, while having a low solubility, are still capable of sufficiently fast dissolution in gastric conditions (i.e., pH 1–3) (Rohner et al., 2007; Wegmüller et al., 2004). Furthermore, as iron-pyrophosphate salts (FePP_i) are white, colloidal particles of this material should be easy to conceal in various food products (van Leeuwen, Velikov, & Kegel, 2012c).

In order to further decrease the reactivity of the contained iron, a second dietary mineral such as calcium or magnesium was incorporated. With this, it was intended to dilute the (surface) concentration of iron in the particles and further reduce its reactivity. An added benefit of these mixed systems is that combining iron with other dietary minerals would make the resulting particles a multi-purpose, widely applicable delivery system for micronutrients (Hilty et al., 2010; Mehansho, Mellican, Hughes, Compton, & Walter, 2003).

Finally, the colloidal particles were coated with zein, a water insoluble prolamin-class protein from corn. A layer of this hydrophobic protein could help to protect the iron. The protein can then be digested in the gastric tract, releasing its contents which can be dissolved and absorbed.

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To summarise, the three systems analysed are (a) Fe^{3+} -pyrophosphate 2, (b) mixed-metal pyrophosphates containing a second cation, either monovalent (Na⁺) or divalent (Mg²⁺ or Ca²⁺, together M²⁺), and (c) an inorganic-protein composite system.

To investigate the reactivity of these systems, gallic acid (GA) was used as a model system for the polyphenols present in foods products (Chvátalová, Slaninová, Březinová, & Slanina, 2008; Fazary, Taha, & Ju, 2009). The formation of the Fe³⁺–GA complex can be followed over time using spectrophotometry, as the complex has a dark blue colour (Chvátalová et al., 2008; Mellican et al., 2003). This increase in absorption was used as an indication for the reactivity of the iron contained in the particles. However, the analysis is complicated by the ability of polyphenols to reduce Fe³⁺, resulting in a Fe²⁺-quinone complex that is also blue. Although various possible pathways are known for this reaction (Arif Kazmi, Sagib Oureshi, & Magsood, 1987; Funabiki et al., 1986: Powell & Taylor, 1982), the most probable one under physiological conditions is described by Hynes (2001). Once the quinone has been formed, the Fe^{2+} can be oxidised to form a new complex with free gallic acid. As will be shown here, the oxidation reaction is much slower than the initial complex formation and the cyclisation of the reaction can be limited by sealing the sample air tight. The difference between the two complexes can be distinguished using spectrophotometry, since they have different absorption maxima, although it does interfere with the quantification of the complexation reaction. Due to the side reactions and the complexity of the system, only the initial reactivity during the first 5 h after addition was analysed and only qualitative comparisons between identically prepared samples were made.

2. Methods

2.1. Materials

FeCl₃·6H₂O (ACS reagent grade, 97%) and zein protein were obtained from Sigma Aldrich. Na₄P₂O₇·10H₂O (ACS reagent grade), CaCl₂·2H₂O (ACS reagent grade, \geq 99%) and NaCl (p.a., \geq 99.5%) were purchased from Merck and MgCl₂·6H₂O (puriss. p.a., \geq 99%) from Fluka. Gallic acid (extra pure, \geq 99.5%) was obtained from Scharlau Chemie. All chemicals were used as received; aqueous solutions were prepared using water deionised by a Millipore Synergy water purification system. Systems were dialysed using Spectra/Por 2 Dialysis Membrane, molecular weight cut-off (MWCO) 12–14 Da, corresponding to roughly a 1.5 nm pore size.

2.2. Iron pyrophosphates prepared by coprecipitation

Iron pyrophosphate was prepared as described previously (van Leeuwen et al., 2012a, 2012c). Briefly, nanoparticles were prepared by coprecipitation of $Na_4P_2O_7$ with FeCl₃. 0.86 mmol iron chloride dissolved in 50 ml water was added drop wise, over about 15 min to 0.64 mmol sodium pyrophosphate in 100 ml. A turbid white precipitate formed in the final 5 min of the addition (van Leeuwen et al., 2012c), the resulting dispersion had a pH of 4.

2.3. Iron pyrophosphate prepared by pH-dependent precipitation

The pH-dependent preparation comprised of two steps: first, the precipitation and washing of the intermediate pyrophosphate salt, which was subsequently redissolved in acid and then precipitated in an alkaline solution. For the intermediate, 50 ml 1 M M^{2+} Cl₂ solution was added drop wise over 1 h to 800 ml 0.0625 M sodium pyrophosphate solution while stirring with a magnetic stirrer. Stirring was continued for another hour after complete addition. The resulting white suspension was washed three times

with water by centrifugation and twice with acetone. Finally, the precipitate was dried in an oven at 37 °C for 2 days.

For the final dispersion, 0.56 g of the intermediate was dissolved in 15 ml 1 M HCl and filtered over a Minisart disposable cellulose acetate filter (0.2 μ m pore size, 16534-K). The solution was injected into 35 ml 0.39 M NaOH solution while stirring vigorously with a magnetic stirrer. The turbid white dispersion was stirred for another 10 min after injection, the pH of the final dispersion was 7. The sample was washed twice by centrifugation and redispersed in a final volume of 50 ml water.

2.4. Mixed systems containing Fe^{3+} and either Na⁺, Mg²⁺ or Ca²⁺

It has been shown previously that the stability of metal-pyrophosphate dispersions is strongly dependent on the ionic strength of the solution (van Leeuwen et al., 2012a). Therefore, mixed systems at a fixed concentration of pyrophosphate were prepared as this set the concentration of the counterions. Mixed systems were prepared by substituting part of the iron in the precursor solution with calcium or magnesium (together referred to as M^{2+}), the amounts of Fe³⁺ and M²⁺ in the mixture are then determined in stoichiometry with the concentration of PP_i. This resulted in the following Fe:M²⁺ ratios: Fe₁₀M²⁺PPi₈ (10:1 ratio), Fe₁₆ M²⁺₂PP_{i13} (8:1), Fe₈ $M^{2+}_{2}PP_{i7}$ (4:1), Fe₄ $M^{2+}_{4}PP_{i5}$ (1:1), Fe₂ $M^{2+}_{11}PP_{i7}$ (1:5) or Fe₂M²⁺₁₉PP_{i11} (1:10). Here complete precipitation without inclusion of the Na⁺ and Cl⁻ from the reactants was assumed. Iron pyrophosphate prepared without any substitution was referred to as 'pure FePP_i'. Full substitution of iron results in the pure M²⁺ pyrophosphate, M²⁺PP_i.

For Fe:Na, the following ratios were prepared: $Fe_{22}Na_2PPi_{17}$ (10:1), $Fe_{32}Na_4PP_{i25}$ (8:1), $Fe_{16}Na_4PP_{i13}$ (4:1). Samples containing a lower iron content remained clear and no particles were formed. All samples were stored in plastic (TeflonTM) bottles. Mixed systems prepared using the pH dependent precipitation method only resulted in stable dispersions when prepared using Magnesium.

2.5. Inorganic-protein composites

Colloidal (mixed) iron pyrophosphates were coated with zein protein through an antisolvent precipitation method (Velikov & Pelan, 2008). As colloidal iron pyrophosphate aggregates over time in water (van Leeuwen et al., 2012a), the nanoparticles were prepared either immediately before (in case of the NP-Z system) or simultaneously with the zein precipitation. The concentrations were also lowered: the final dispersion contained 2 mM iron and 1.5 mM pyrophosphate, in order to prevent aggregation during the addition of zein. After complete precipitation of the iron pyrophosphate, the 30 ml dispersion was removed and 40 ml zein solution (1 g zein in 80 vol.% ethanol) was slowly poured into the dispersion, which turned more turbid and slightly yellow. Some aggregates were formed, which were filtered out of the dispersion before further analysis.

2.6. Analysis

Dynamic Light Scattering (DLS) measurements were performed on a Malvern Instruments Zetasizer Nano series machine in backscatter mode at 25 °C with 5 min of equilibration time. As the formed clusters and particles were polydisperse (>30% in some cases), cluster sizes derived from DLS are only interpreted as trends (van Leeuwen et al., 2012b). Samples were dried on a carbon-coated copper grid prior to transmission electron microscopy (TEM) performed on a Tecnai 12 or scanning electron microscopy (SEM) using a Phenom scanning electron microscope, both from FEI Company.

2.7. Spectrophotometry

All samples for spectrophotometry were prepared to contain the same concentration of iron (0.7 mM). Samples were diluted to the correct concentration prior to analysis. All systems were at or close to pH 5 after dilution. Excess gallic acid (3.5 mM) was added and the cuvette sealed air-tight for spectrophotometry using a Perkin-Elmer Lambda-35 spectrophotometer. Samples were thermostated at 23 °C and magnetically stirred during spectrophotometry. The influence of (a change in) sample turbidity on the absorbance was countered by using the dispersion at the same concentration but without gallic acid as reference. The gallic acid addition and vial sealing could not be done inside the spectrophotometer while the measurement was running. Therefore, the blanks were placed first and the samples with gallic acid were then prepared in quick succession. No more than two samples with gallic acid were analysed during a single experiment, so that the time between addition of gallic acid and the first measurement was never more than a few seconds.

3. Results

3.1. Particle preparation and colloidal stability

The preparation of metal pyrophosphate particles by coprecipitation of the precursor salts has been previously investigated (van Leeuwen et al., 2012a, 2012c). While this method resulted in stable colloidal dispersions of iron pyrophosphate (FePP_i), it was shown that pyrophosphate coprecipitated with a divalent metal ($M^{2+}PP_i$) in general formed particles that were too large to remain in suspension. Furthermore, stable dispersions of mixed systems were only prepared at a high iron content (>80%) (van Leeuwen et al., 2012c), while a lower iron content was preferable in order to reduce the reactivity of the contained iron. Preparation by coprecipitation of pure FePP_i or mixed systems at a low M (Na or M^{2+}) content resulted in clusters of small, amorphous particles, shown in Fig. 1a and observed previously (van Leeuwen et al., 2012c). The FePP_i-zein preparation method yielded polydisperse particles of around 150 nm containing the insoluble salt as can be observed in Fig. 1b. An empty zein particle is shown for reference in Fig. 1c.

Due to the fact that coprecipitation by slow addition is an ill-defined method of preparation, this study also used pH-dependent precipitation as a more controlled way of preparing $M^{2+}PP_i$ particles. $M^{2+}PP_i$ s are soluble at a low pH (Haynes, 2011), which can be used for a two-step preparation method in which the material is first coprecipitated, washed and dried and finally re-dissolved in acid. The pH of the solution is then raised again to induce precipitation.

Using this method, stable dispersions of magnesium pyrophosphate were prepared. Calcium resulted in particles too large and aggregated to remain in dispersion (Fig. 1d). The same held for the mixed systems: only mixed systems containing magnesium and less than 5% Fe³⁺ resulted in stable colloidal particles (see Supplemental Material Table S1 for details). Morphologically, all magnesium containing systems looked similar. From TEM analysis, it was found that small, thin, irregular platelets of about 50 nm were formed (Fig. 1e and f).

3.2. Long-term stability

Fig. 2 shows that the zein coated systems and Mg-containing systems prepared by the pH-dependent precipitation method remained stable for much longer periods of time compared to pure FePP_i. The Mg-containing mixed systems remained stable for more than four months (Fig. 2c), further washing steps did not improve dispersion stability for any of these systems (not shown).

The mixed systems prepared by coprecipitation at an Fe content above 80% had a stability similar to the pure FePP_i, although the amount and type of secondary metal used had a great influence on this stability (Fig. 2b–d). The relative stability was clearly influenced by the cation used; Ca²⁺ substituted systems destabilised within days, while Na⁺ substituted systems remained stable for over three months. There appeared to be no specific order in the effect of the substitution ratio as it varied per substituting metal.



Fig. 1. TEM images of the systems prepared and analysed, by coprecipitation: FePP_i. (a) and FePP_i coated with zein (b) an empty zein particle (c) samples prepared by pH-dependent precipitation: CaPP_i (c), MgPP_i (d) and the Fe:Mg 1:50 mixed system (e). Mixed systems containing Na as the additional cation are identical to those shown in Figure a (not shown).



Fig. 2. Growth of cluster size over time as analysed by Dynamic Light Scattering. (a) DLS analysis shows the improved stability of the Mg-substituted system and the zeincoated system over the pure FePP₁ prepared by coprecipitation. (b–d) Systems prepared by coprecipitation containing different ratios of metal pyrophosphates. Calcium (a), magnesium (b) and sodium (c) systems with Fe:M ratios of 10:1 (dark grey squares), 8:1 (diamonds) and 4:1 (triangles). Pure FePP₁ shown for reference (stars).

3.3. Spectrophotometry

An initial test reaction demonstrated the clear inhibition of the Fe–GA complex formation by incorporating the iron in an inorganic matrix. Fig. 3a–e shows that a solution of FeCl₃ sample immediately turned black upon the addition of gallic acid while a sample containing iron pyrophosphate had only reached full colouration after seven days. Analysis by spectrophotometry (Fig. 3f and g) showed that most of the complex formation occurred within the first hour and that the quinone signal at 395 nm started to become significant after about 4 h, making further analysis of the reaction inaccurate. Therefore, it was decided to analyse the absorbance at 560 nm only for the first 5 h after the addition of gallic acid.

3.4. Reactivity of colloidal iron pyrophosphate

Spectrophotometric analysis of the complex formation over time showed a clear influence of the preparation method on

the reactivity of the particles. A sample freshly prepared by the coprecipitation method increased absorbance until it reached its maximum value after about 60 min (Fig. 4a), while the dialysed system increased much more slowly and had not fully reached its plateau value after 300 min. A solution of FeCl₃, at the same concentration of iron, had an initial absorbance of 0.8 (not shown), indicating successful protection of the majority of the Fe³⁺ at least for the duration of the analysis. Fig. 4a also shows that ageing a system for one month before the addition of gallic acid further decreased its reactivity. This cannot be due to the larger cluster size caused by ageing (as shown in Fig. 2), as a dialysed system that was aged for one month also showed the lowered reactivity while the clusters had not grown (see Supplemental Material Table S2 for more details). It might be due to the disappearance of the smallest particles after dialysis or ageing that has been reported previously (van Leeuwen et al., 2012b). Just after preparation by coprecipitation, colloidal FePP_i consisted of 200 nm clusters of larger particles, and indi-



Fig. 3. Initial reactivity studies. Top row of photos shows the inhibited reactivity of FePPi compared to $FeCI_3$ at same concentration of iron (a–e). While $FeCI_3$ turns black instantaneously upon addition of gallic acid (a), a freshly prepared dispersion of $FePP_i$ (b), immediately (c), 4 h (d) and 8 days (e) after addition of gallic acid shows the slow discolouration of the dispersion. UV–Vis spectra of the reaction mixture up to 4 h (f) and up to 7 days (g) after addition of gallic acid show the fast initial complex formation at 560 nm, and the slower reaction of quinone formation at 395 nm.



Fig. 4. Iron-gallic acid complex formation followed over time in pure FePP_i samples (a) and mixed systems (b), all prepared at the same concentration of Fe³⁺ (0.7 mM). The absorbance of FeCl₃ with gallic acid at this concentration is 0.8 mM (not shown). Pure FePP_i ("Fresh") is added to (b) for reference. TEM images of pure FePP_i before (c) and after (d) the reaction with gallic acid shows a smoother particle surface after the reaction. The Fe:Mg 1:50 mixed system 5 h after the addition of gallic acid: (e) without GA and (f) with GA. While the GA-containing dispersion has aggregated, the sample does not show the colouration characteristic of the Fe–GA complex seen in Fig. 3.

vidual nanoparticles of around 5 nm. After dialysis or ageing, these individual particles were no longer present. As smaller particles have a higher solubility, the lack of these particles in a system could explain the lowered reactivity observed in Fig. 4a.

It is interesting to note that while both the freshly prepared and freshly dialysed systems started at the same initial value at t = 0, this initial jump was much smaller for the aged systems. The initial jump at t = 0 indicates that a large part of the reaction occurs at the surface of the particles, while the decrease with ageing seemed to indicate that the surface reactivity somehow lowered over time. The origin of this surface passivation is currently unknown. Here it should be noted that t = 0 is not actually the moment that the gallic acid was added, but the moment the measurement was started. This was a few seconds after the addition of gallic acid (see Section 2 for details). As this was a much shorter timeframe than the initial increase of absorption in Fig. 4a, this cannot be the origin for these initial jumps.

Table 1 shows no obvious change in the cluster size and zetapotential due to the reaction with gallic acid, and the increased conductivity was due to the addition of gallic acid. TEM analysis (Fig. 4c and d) showed that the surface of the particles had become somewhat smoother after the reaction, possibly due to dissolution. The conductivities of the samples showed a significant decrease after dialysis.

Comparing the data of Fig. 4a to that in Table 1, the initial jump in the absorbance and conductivity of the non-dialysed systems both cannot be caused by residual Fe³⁺ in solution. This is because the fresh and dialysed fresh samples in Fig. 4a had an identical initial jump, while the conductivity was two orders of magnitude lower as shown in Table 1. A conductivity of 2.2 mS/cm would correspond to around 20 mM NaCl (McCleskey, 2011), which was in good agreement with the 17 mM added during preparation.

Filtering the suspensions prior to analysis by spectrophotometry in order to reduce noise levels and exclude surface effects during the reaction was also attempted. Unfortunately, the systems contained small, 5 nm particles that remained in the dispersion and could not be filtered out(van Leeuwen et al., 2012b), rendering more accurate analysis impossible.

3.5. Reactivity of mixed systems

3.5.1. Inorganic-protein composite systems

The data of the zein-coated pure $FePP_i$ (Fig. 4b) showed that coating the particles with zein reduced the reactivity of the contained iron. However, the data also showed one important disadvantage of this analysis method: the high turbidity of these dispersions drastically increased the noise level.

3.5.2. Mixed systems with high Fe^{3+} content

As can be seen in Fig. 4b, the reactivity of the mixed systems with a high iron content was higher than the reactivity of the pure FePP_i as the initial slopes were steeper and the final absorbances higher. The most stable mixed system was used for every cation: 4:1 for Na, 8:1 for Mg and 10:1 for Ca (see Fig. 2b–d). The reactivity of the dispersions increased with the stability of the dispersion. While the Ca mixed system completely aggregated within days, its reactivity was closest to that of pure FePP_i. On the other hand,

able I			
luster size, zeta-potential (ZP) and	d conductivity (C) before and	l after reacting 5 h wi	th gallic acid.

	Size (nm)	ZP (mV)	St.Dev. (mV)	C (mS/cm)	St.Dev. (mS/cm)
Fresh	215	-32	0.5	2.10	0.09
Diluted ^a	215	-35	0.4	0.35	0.007
Aged 5 h	210	-34	0.7	0.34	0.006
Aged with GA	210	-33	0.5	0.51	0.012
GĂ	-	-	_	0.15	0.003

^a Diluted to the concentration used for spectrophotometry, 0.7 mM Fe³⁺.

the Na system was the most reactive of all the compounds tested here, while it remained stable in dispersion for months.

3.5.3. Mixed systems with high M^{2+} content

As mentioned, it was only possible in this study to prepare stable colloidal systems at high M^{2+} content using magnesium. Systems containing Ca sediment within minutes to hours while Na containing systems did not form particles at all. However, it has not been possible to analyse the reactivity of Fe:Mg mixed systems with a low iron content. The addition of gallic acid caused the dispersion to aggregate completely, as shown in Fig. 4f with a Fe:Mg 1:50 dispersion. The figure also shows that there was no appreciable discolouration for up to 5 h after the addition of gallic acid, indicating that the contained iron was successfully protected from reaction. As discussed in the Section 2, it was not possible to prepare particles at a Na content higher than 4:1 as the resulting mixture contained no particles.

4. Conclusions

Stable colloidal dispersions of various (composite) pyrophosphates containing iron have been prepared. While the pure FePP_i system destabilised over time, coating the particles with zein protein or substituting the majority of the iron with magnesium resulted in systems that remained stable for months. Using the complex formation of iron with gallic acid as a model system for the reactivity of Fe³⁺ in foodstuffs, it has been shown that embedding the iron in an inorganic matrix reduces its reactivity relative to FeCl₃. Analysis of the aged and dialysed systems indicated that most of the reactivity decreased over time. Coating the particles with zein successfully protected the incorporated iron as it further decreased its reactivity.

It was shown that mixed systems actually increased the reactivity at low iron content. There was a counterintuitive trend for the mixed systems, in the sense that the less iron the particles contained, the more reactive they became for Fe:M ratios below 4:1. However, at a much lower iron content (below 5%), the reactivity decreased drastically as no discolouration was observed with of the Fe:Mg 1:50 mixed system after 5 h.

It was found that while the gallic acid method was useful to determine the relative reactivity of iron-containing materials, the side reaction made the method hard to control and inaccurate over longer periods of time. Moreover, due to the colloidal size of the particles there was significant interference with the analysis method, especially when the particles aggregated in the presence of gallic acid, as shown in Fig. 4f.

Finally, while unstable in the presence of gallic acid, the Fe:Mg 1:50 system did not show any appreciable colouration for up to 5 h. This shows that preparing a mixed insoluble salt can reduce the reactivity of one of its components.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014. 01.045.

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