# Crystal Structure of Ferrihydrite Nanoparticles Synthesized in Ferritin

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In this study, horse spleen apoferritins were induced to form biominerals using up to 3000 Fe atoms per protein molecule. The morphology and crystallinity of the nanometer-sized biominerals formed in the ferritins were then analyzed using field emission-energy filtering-transmission electron microscopy (FE-TEM). The ferritins were found to have reconstitution yields of 60-70% in the experiments. The mean core size of the ferritins varied somewhat with protein concentrations, indicating that crystal growth in ferritins could be controlled *via* protein concentrations. The core mineral size increased with the amount of Fe used. Lattice fringes of the core, associated with good crystallinity, were found in all samples. The lattice fringe images of a single domain ferrihydrite mineral appeared frequently in the (011) planes ($d$-spacing of 0.246 nm) under [100] zone axis in all samples of this study. In addition, the lattice image occasionally revealed fringes corresponding to the (100) planes ($d$ = 0.254 nm) from the [001] zone axis, indicating the characteristic pattern of hexagonal crystal lattice. Diffraction patterns in the minerals identified as ferrihydrite were fitted well into the space group of $P3_1c$.

**Key Words :** Ferritin, Ferrihydrite nanoparticles, FE-TEM

## Introduction

Biomineralization is a field built on understanding the structure of minerals made *via* either matrix-control or biological control.[1] The matrix includes many macromolecules that make up a framework in which the minerals form. A remarkable variety of inorganic nanoparticles synthesized in bio-molecular and protein cages has been created and applied in the field of nanotechnology.[2] Protein cages with nanometer-sized cavities provide reaction environments controlling the size, specific shape, chemical composition, crystal morphology and crystal structure of the biominerals being produced. One such cage-shaped protein, ferritin, has been extensively studied.[3-7] Ferritin is a spherical, iron-binding protein with mean diameter 12 nm, composed of 24 polypeptide subunits encapsulating hydrous ferric oxide mineral. Native ferritin generally contains less than 2000 Fe atoms per protein molecule, although it has been known to accommodate up to 4500 Fe atoms.[8,9] The core mineral ferrihydrite ($5Fe_2O_3{\cdot}9H_2O$) of animal ferritins has been studied in detail.[10-12] The protein shell apoferritin is often used to synthesize inorganic nanoparticles such as $Fe_2O_3$,[3] MnOOH,[4] CoOOH,[5] and $Co_3O_4$.[6]

Electron microscopic studies of native and reconstituted ferritins have progressed with the development of high resolution techniques such as X-ray diffraction and TEM. The results of such studies have suggested that the core of ferritin contains a single crystalline or polycrystalline structure within the protein shell.[6,7,13,14] Recent electron nanodiffraction experiments with a beam of 1 nm diameter revealed that the ferritin core contains a single phase with amorphous structure or partially crystalline.[13,14] However, the structure confirmation either way has not been confirmed because homogenous nanoparticles were not formed during the reconstitution of ferritin. Therefore, the structural characteristics of the ferrihydrite nanoparticles in apoferritin should be clarified.

Previously, ferritins were successfully reconstituted with up to 2000 Fe atoms, resulting in variable reconstitution yields (37-72%).[7,15] Reconstitution yields varied depending on the various factors including ionic strengths, reaction volumes and protein concentrations. In the present study, horse spleen ferritins were reconstituted with up to 3000 Fe atoms per protein molecule. The effects of reaction factors of protein concentration and Fe amount were examined in the reconstitution experiments. Then, the morphology and crystallinity of the nanometer-sized biominerals in the ferritins were analyzed *via* field emission-energy filtering-transmission electron microscopy (FE-TEM) and electron diffraction.

## Materials and Methods

Horse spleen ferritin (HSF) was purchased from Sigma. Apoferritins from the ferritins were prepared by chemical reduction using a previously described method.[15] Next, the protein concentration was determined using a modified version of the Lowry method.[16] The apoferritin was then reconstituted adding freshly prepared 40 mM ferrous ammonium sulfate to 40 mM 3-[N-morpholino] propane sulfonic acid (MOPS) buffer (pH 7.0) by the method of Wade *et al.*[17] In brief, $Fe^{2+}$ was added to apoferritin (reaction volume 3 mL) in 40 mM MOPS buffer (pH 7.0). The reconstitutions were carried out in two-different protein concentrations (0.25 mg/mL and 0.5 mg/mL). The $Fe^{2+}$ additions were

made in four steps at RT with 45-min intervals in between each step to give 2000 Fe atoms per protein molecule. At each step 500 Fe atoms per protein molecule were added. For the reconstitution using 3000 Fe atoms per protein molecule, six steps of the $Fe^{2+}$ additions were made. The reaction product was then stored at 4 °C for 18 h, after which it was subjected to dialysis against $H_2O$. The reconstituted ferritin was then centrifuged (5000 ×g, 10 min, 4 °C) to remove the aggregates that might have formed during reconstitution. Upon reconstitution, the content of iron in the ferritin core was analyzed by atomic absorption spectrometry (AAS; SpectrAA-800, Varian) at the Center for University-wide Research Facilities, Chonbuk National University. The reconstitution yield was calculated as the percentage of the iron ($\mu$g) incorporated by the proteins per total iron ($\mu$g) reacted. In addition, unstained samples of the reconstituted HSF were prepared for electron microscopy by placing small drops of the solution onto Formvar-coated copper grids and allowing them to air-dry. The electron microscopic images were then obtained using a JOEL JEM 2200 FE-TEM operating at 200 keV at the Korea Basic Science Institute (Chonju Branch). FE-TEM has been extremely useful providing high contrast by energy filtering for the structures of individual ferritin cores, which allowed dense areas of the ferritin cores selected for further analysis of their electron diffraction patterns. The particle sizes of such areas were then determined based on the sizes of 100 iron cores observed in enlarged photomicrographs.

## Results and Discussion

This study examined the Fe content and its core structure in ferritins reconstituted with up to 3000 Fe atoms per protein molecule. Previously, we could not succeed with more than 2000 Fe atoms and get precipitation upon over the amount. In the present study, the reaction volume of 3 mL in 40 mM MOPS (pH 7.0) at the protein concentrations of 0.25 and 0.5 mg/mL was used. The iron content and reconstitution yield after reconstituting the apoferritin with $Fe^{2+}$ ions are shown in Table 1. Reconstituted with 2000 Fe atoms, the ferritin's iron contents were 1393 ± 37 and 1323 ± 74 Fe atoms per protein molecule for protein concentration 0.25 and 0.5 mg/mL, respectively. In particular, the yield 66.2% obtained from the protein concentration 0.5 mg/mL is similar to our previous work.[7] With 3000 Fe atoms, they turned out to 1819 ± 73 and 1786 ± 68 Fe atoms for 0.25 and 0.5 mg/mL, respectively. These results suggest that the reconstitution yields were not related to the concentrations of protein in 40 mM MOPS buffer. Taken together, the yields indicate that 60-70% of the Fe atoms remained in the core, while the rest may exist as non-specific precipitation. It implies that $Fe^{2-}$ oxidation by the dissolved oxygen in the solution is extremely fast and the precipitation occurs outside the protein. Such fast reaction rates had also been observed in the iron uptake experiments using HSF, human H- and L-chain ferritins.[7,18,19] However, it could be possible to reduce the $Fe^{2-}$ oxidation and reaction rate by controlling oxidizing agents under anaerobic conditions at least *in vitro*.[9] Previously, Meldrum *et al.*[20] had prepared the reconstituted ferritin with Fe, but its reconstitution yield was not shown.

**Table 1.** Reconstitution yields of ferritins with 2000 and 3000 Fe atoms per protein molecule. The reconstitutions were carried out at protein concentrations 0.25 and 0.5 mg/mL

| Theoretical Amount | Protein conc. (mg/mL) | Analytical Amount [a] | Yield (%)[b] |
|---|---|---|---|
| 2000 | 0.25 | 1393 = 37 | 69.7 |
| | 0.5 | 1323 = 74 | 66.2 |
| 3000 | 0.25 | 1819 = 73 | 60.6 |
| | 0.5 | 1786 = 68 | 59.5 |

[a]Values represent Fe atoms in ferritin core. [b]The yield was calculated as the percentage of the iron ($\mu$g) incorporated by the proteins per total iron ($\mu$g) reacted.

The representative electron photomicrographs of the unstained images of the native and reconstituted HSF are shown in Figure 1. The images of the discrete electron dense cores indicate that the reconstitutions went well, as in our previous studies.[7,15] The negatively stained images of the reconstituted cores of ferritin revealed that they were generally spherical in shape and enveloped by polypeptide shells (data not shown). The core size of the ferritin reconstituted at protein concentration 0.25 mg/mL was found to have greater variation than ferritin reconstituted at 0.5 mg/mL under the same conditions for Fe atom reaction. When the Fe content was increased from 2000 to 3000 atoms, the

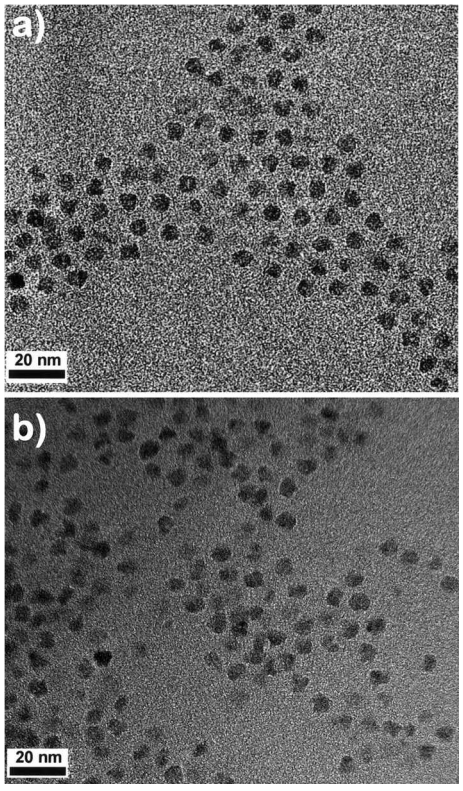


**Figure 1.** Transmission electron micrographs of the iron cores of ferritins. (a) Ferritin reconstituted with 3000 $Fe^{2-}$ ions at the protein concentration 0.5 mg/mL; (b) native HSF.

**Table 2**. Particle size data of the reconstituted ferritins

| Theoretical Amount[a] | Protein conc. (mg/mL) | Particle Mean (nm) | Particle Range (nm) |
|---|---|---|---|
| 2000 | 0.25 | 5.2 | 2.9-7.3 |
| | 0.5 | 6.5 | 4.7-8.6 |
| 3000 | 0.25 | 6.2 | 3.6-8.1 |
| | 0.5 | 6.7 | 4.8-8.6 |

[a]Values represent Fe atoms added to apoferritin.

core appeared to remain generally spherical with increasing size (data not shown). The images of the native HSF cores were also spherical, although some cores appeared to be partially-filled in (Fig. 1). The iron contents obtain from these experiments were less than the native HSF value of 1960 ± 70 Fe atoms.[15]

Table 2 and Figure 2 show the size distribution of the reconstituted ferritins under different conditions. Reconstituted with protein concentration 0.25 mg/mL and 2000 or 3000 Fe atoms, the core size ranged 2.9-7.3 nm (mean 5.2 nm) or 3.6-8.1 nm (mean 6.2 nm), respectively. In contrast, with protein concentration 0.5 mg/mL and 2000 or 3000 Fe atoms, reconstituted ferritin core size ranged 4.7-8.6 nm (mean 6.5 nm) or 4.8 to 8.6 nm (mean 6.7 nm), respectively. It is to be noted that the Fe amount reacted (2000 and 3000 atoms) had no much effect on the ferritin core size at the higher protein concentration 0.5 mg/mL but drastic effect at the lower 0.25 mg/mL. Present result for 3000 Fe atoms and protein concentration 0.5 mg/mL is consistent with an earlier report (3000 atoms and 0.5 mg/mL).[20] Thus, the ferritin core size increment may indicate that the crystal grows preferentially in the core with increasing Fe amounts and protein concentration. It means that the crystal growth in the ferritin core can be controlled by varying Fe amounts or protein concentrations, which is consistent to our previous study.[7]

Lattice images of single domain ferrihydrite crystals of the reconstituted ferritin cores are shown in Figure 3. The lattice

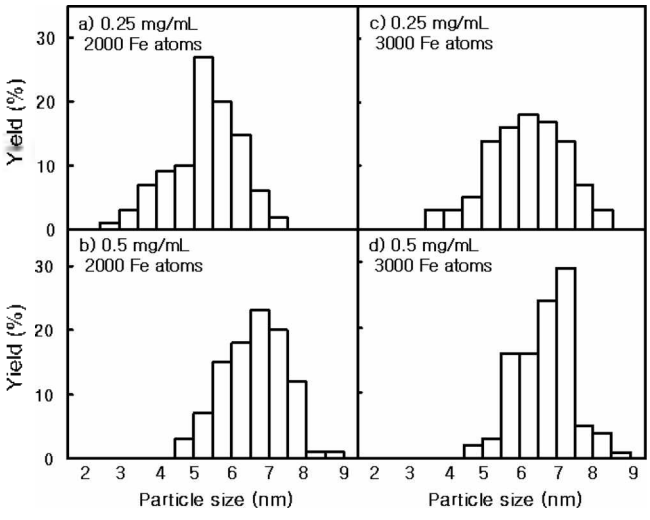


**Figure 2**. Particle size distributions of the ferritins reconstituted with 2000 and 3000 Fe atoms at protein concentrations 0.25 and 0.5 mg/mL.

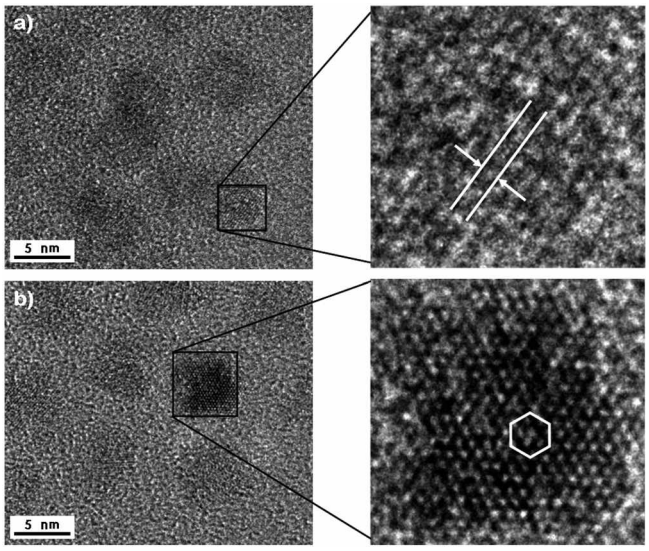


**Figure 3**. High-resolution lattice images of the reconstituted ferritin cores at the protein concentration 0.5 mg/mL. (a) Lattice fringes of the (011) planes (0.246 nm) of ferrihydrite produced with 2000 Fe atoms; (b) Lattice fringes of the (100) planes (0.254 nm) of ferrihydrite produced with 3000 Fe atoms.

fringes of the ferritin core reconstituted in this study are almost visible. They have low contrast against the carbon substrate background; however, an intensity modulations of the images led to better resolution within the individual crystallinities. Lattice fringes of the (011) planes (*d*-spacing 0.246 nm) under the [100] zone axis were dominant (Fig. 3a). In addition, dotted patterns were occasionally obtained in some places in the (100) planes (0.254 nm) under the [001] axis, indicating presence of three dimensional structural feature (Fig. 3b). Single crystalline cores were observed for particles with dimensions 5.0-7.0 nm. Overall, these findings are similar to the result of a previous study.[21] Lattice fringes created by interference of the electron beams that are diffracted through the lattice of the core were commonly found when high concentrations of protein (0.5 mg/mL) were reacted with 3000 Fe atoms per protein molecule (Fig. 3). Such features are closely associated with good crystallinity of the sample, although using the presence of a lattice to indicate the preferred orientation of a single domain ferrihydrite crystal should done with caution.

Selected-area electron diffraction obtained from native ferritin consisted of five ring patterns with *d*-spacings of 0.247, 0.234, 0.198, 0.173 and 0.148 nm, as shown in Figure 4. The diffraction intensity of the mineral appeared strongly in the (011) plane (0.247 nm) and the (120) plane (0.148 nm). It is somewhat in good agreement with synthetic six-line ferrihydrite reported by Janney *et al.*[22] In addition, the diffraction patterns of the reconstituted core minerals that were identified as ferrihydrite are well fitted into the space of $P3_1c$.

A previous study conducted by Meldrum *et al.*[20] indicated that the crystallinity (or lattice fringe image) of reconstituted ferritin was lower than that of native HSF. However, the results of the current experiment revealed no difference

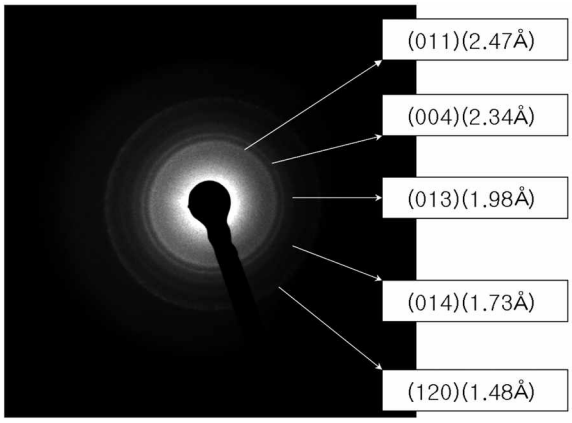


**Figure 4**. Electron diffraction patterns of the native HSF. The *d*-spacings (0.247, 0.234, 0.198, 0.173 and 0.148 nm) of ferrihydrite are shown.

between the reconstituted and native HSFs. This discrepancy may have occurred due to differences in sample handing and the conditions of the experiment.

In summary, we successfully conducted artificial synthesis of ferrihydrite nanoparticles in the cavity of horse spleen apoferritin. The minerals were induced using up to 3000 Fe atoms per protein molecule. The shape, size distribution and crystallinity of crystals in the ferritin core were determined using FE-TEM and could be controlled by changing the protein concentrations and iron amounts. The diffraction patterns of the ferrihydrite nanoparticles fit well into the space group of $P3_1c$.

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