Synthesis of Co_3O_4 Nanoparticles Using the Cage-Shaped Protein, Apoferritin

Rikako Tsukamoto,[1] Kenji Iwahori,[1] Masahiro Muraoka,[1] and Ichiro Yamashita*[,1,2,3]

[1]CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012

[2]Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma 630-0192

[3]Advanced Technology Research Laboratories, Matsushita Electric Industrial Co., Ltd., Hikaridai, Seika, Kyoto 619-0237

Received June 1, 2005; E-mail: ichiro@ms.naist.jp

The one-pot synthesis of cobalt (Co) oxide nanoparticles in the apoferritin cavities was studied. A detailed survey revealed that the optimum conditions are mixing 3 mM Co(II) ion with 0.5 mg/mL apoferritin in 100 mM HEPES pH 8.3 buffer solution followed by the oxidation of Co(II) ion by the addition of hydrogen peroxide (H_2O_2) at 50 °C. Under these optimum conditions, Co oxide cores were formed in almost all the apoferritin cavities in a spatially selected manner. The selection of the buffer reagent was critical to synthesizing the Co oxide cores and the elevated temperature was effective for the fast synthesis. X-ray photoemission spectroscopy (XPS) and electron energy-loss spectroscopy (EELS) proved that the cores contain cobalt atoms. X-ray powder diffraction (XRD) structure study revealed the core structure as Co_3O_4, which was consistent with the lattice images of the cores observed by high resolution TEM. The biological process presented in this paper provides a simple and mass-producible method for producing homogenous Co_3O_4 nanoparticles.

Nanoparticles (NPs) are attracting researchers' interest as the key material of nanotechnology. NPs are already used or expected to be used in many fields, such as magnetic recording materials, catalytic materials, fluorescent markers, drug delivery systems, and quantum electronics. Especially, conductive or semiconductor NPs are anticipated to be good quantum electronics device components. For this application, the size of the NP is critical, because the electron energy levels are strongly dependent on the NP size. Consequently, homogeneous NPs are required to satisfy this requirement. It has thus been proposed that the cavity of the cage-shaped protein, apoferritin, should be used for the NP synthesis. Since the protein molecules have an atomically identical structure, the NP synthesized in the cavity should have the same diameter.

Apoferritin is an iron storage cage-shaped protein which is present in many biological species. When iron ions are sufficient, iron atoms are stored in the cavity as an iron hydroxide core in vivo.[1,2] Figure 1 is a schematic drawing of the apoferritin molecule. The outer and inner diameters are approximately 12 and 7 nm, respectively. There are narrow channels along the three-fold axis connecting the cavity and outside, through which iron ions penetrate the cavity. The protein shell is composed of 24 subunits and there are two types of subunits, called L-subunit and H-subunit. Only H-subunit has the ferroxidase site, which oxidizes the iron(II) ion and produces the iron(III) hydroxide cores.[1,2] Meanwhile, L-subunits play an important role for the ferritin molecule to self-assemble into a two-dimensional crystal at the air–water interface with a salt-bridge interaction.[3,4] Making use of the same size nanodot core of the apoferritin and the self-assemble ability of the protein shell to make a two-dimensional crystal, together with the selective elimination of the labile protein moiety, we fabricated a nanodot array for a floating gate memory.[5] This biological method was called the Bio Nano Process.

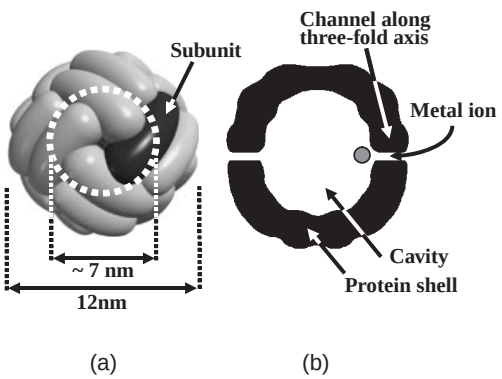


Fig. 1. Schematic drawing of apoferritin molecule. (a) Molecule viewed down a four-fold axis, and (b) cross section including two three-fold channels. The spherical protein shell consists of 24 subunits. One subunit is colored black and the size of the core is shown by the white dashed line circle. The outer diameter of the shell is about 12 nm and the core size is about 7 nm. There are narrow channels connecting the inner cavity and outside, through which metal ions enter the inner cavity. The self-assembled protein cage has the ability to sequester and store iron as a hydrated iron oxide in the internal cavity.

There are references reporting that metal complexes and inorganic materials can be sequestered in the native apoferritin cavity.[6–17] Some researchers reported the core formation in

Published on the web October 31, 2005; DOI 10.1246/bcsj.78.2075

vivo and others reported core synthesis in the native or horse spleen apoferritin (HsAFr) cavity in vitro. The latter include our reports of nickel hydroxide, chromium hydroxide, and cadmium selenide core formations in the HsAFr cavity.[16,17] The sizes of the obtained CdSe cores were measured from a TEM image and the standard deviation of the diameter was less than 10% of the average diameter.[17] For electric device applications, metal oxide NPs are attractive because they can be reduced to conductive metal by several process methods. Cobalt (Co) oxide and hydroxide NPs are among such attractive ones.

Recently, Douglas et al. reported Co(O)OH, cobalt hydroxide, core formation in the HsAFr by oxidizing Co(II) with H_2O_2 while the reaction solution was dynamically titrated at pH 8.5 using NaOH.[14] Allen et al. also made Co_3O_4 cores in the *Listeria ferritin* by dynamic titration.[15] Dynamic titration was successfully used to synthesize a Co oxide core inside the apoferritin cavity. Dynamic titration has a merit that the reaction mixture does not contain additional chemical agents for maintaining solution pH. However, the pH control using a buffer agent is much simpler. The one-pot synthesis using a buffer solution is more suitable for mass-production. In this paper, we report the one-pot synthesis of cobalt oxide NPs in the recombinant apoferritin composed of L-subunits as well as HsAFr using a buffered reaction solution. The effect of the buffer reagents on the Co oxide core biomineralization and the experimental survey of the best chemical reaction conditions of the Co oxide core synthesis in the cavities of both HsAFr and the recombinant apoferritin are described in detail. The characterization of the obtained Co oxide cores is also reported.

Experimental

Commercial and Recombinant Apoferritin. HsAFr was purchased from Sigma and was used as received. The recombinant L-apoferritin (L-AFr) molecules were over-produced using a plasmid pMK2 which carries the DNA sequence of the horse lever L-type subunit lacking eight N-terminus amino acid residues, and Nova Blue was used as a host cell. This recombinant apoferritin was called Fer-8. The bacteria were collected by slow centrifugation and suspended in 50 mM tris(2-amino-2-hydroxymethyl-1,3-propandiol)-HCl pH 8.0 + 150 mM NaCl. The solution was sonicated and heat-treated at 60 °C for 20 min. The solution was then centrifuged at low speed and the supernatant was retrieved. Recombinant apoferritin in the supernatant was purified through an ion-exchange column (Q-sepharose, Amasham Biosciences) and by gel filtration (Hiprep Sephacryl S-300, Amasham Biosciences and G4000SWXL PEEK, TOSOH). The ion exchange column extraction was fractioned and the fractions were checked by SDS-PAGE (SDS: sodium dodecyl sulfate, PAGE: polyacrylamide gel electrophoresis). Only the fractions containing the recombinant apoferritin were collected and processed through the gel filtration. In the gel filtration column, ferritin 24mers was carefully collected excluding the dimers or trimers of the apoferritin molecule. The concentration of the apoferritin protein was adjusted to around 10 mg/mL in 150 mM NaCl and the samples were stored at 4 °C until use.

Survey of the Optimum Solution Conditions for Cobalt Oxide Core NPs Synthesis. A 0.5 mg/mL (1 μM) apoferritin solution was prepared and adjusted between pH 7.3–8.8 with 100 mM buffer agent. A 37.5 mM Na_2SO_4 solution was also added. The solution was stirred by a magnetic stirrer bar and ammonium cobalt sulfate (ACS) was added to create a final concentration of 2 to 5 mM, followed by the addition of H_2O_2 with a half-stoichiometric concentration of ACS. The solution was stirred for about 20 min and then left overnight. The solution was kept at 50 °C using the temperature control aluminum box. The solution after Co NP synthesis was centrifuged at low speed to remove the precipitates. The protein concentration of the supernatant was measured by the Bradford protein assay method.

Transmission Electron Microscopy (TEM) Observation and Electron Energy-Loss Spectroscopy (EELS) Measurement. Four microliters of ferritin solution was placed on the electron microscope carbon grid and left to stand for a minute in order for the ferritin to adhere to the carbongrid. The excess solution was then blotted and the sample was air-dried. Aurothioglucose (2.5%) was used to stain the sample when necessary. Aurothioglucose has been proven not to stain the apoferritin cavity.[16] The ferritins on the carbongrid were observed by TEM (JEM1010 and JEM-2200FS, JEOL) to observe the core formation. The number of core-formed apoferritins was counted and divided by the number of total apoferritin molecules in one TEM image. This ratio was named the core formation ratio (CFR). The yield of the NPs was evaluated by calculating the products of the protein concentration in the solution and CFR.

The cores were also observed without staining at high magnification and lattice images of the cores were obtained (EM-002B, Topcon). EELS measurement of the synthesized cores was carried out and the presence of cobalt atoms in the core was examined (HF-2200, Hitachi).

X-ray Photoemission Spectroscopy (XPS) Measurements. Ferritin molecules after the core synthesis were intensely washed using Milli-Q water. Ferritin molecules in the Milli-Q water were then put on the silicon substrate. The substrate with ferritin solution was placed in an Eppendorf tube and centrifuged to spin down the excessive solution. The obtained sample was used for the XPS measurements (AXIS165, KRATOS). The C1s peak was used as a reference for calibration and all the values of the binding energy were shifted so as to maintain a constant value for the C1s binding energy.

X-ray Powder Diffraction (XRD). X-ray powder diffraction was carried out on the synthesized NPs. In each experiment, several tens of mg of apoferritin with a Co oxide core was extensively washed with Milli-Q water before being dried with flowing dry nitrogen gas. The obtained material was ground to powder using a mortar and pestle. Finally, this powder was used as a sample for the X-ray powder diffraction. Measurements were carried out using an X-ray generator (RINT2500/PC, Rigaku) operated at 50 kV and 200 mA with a Cu target.

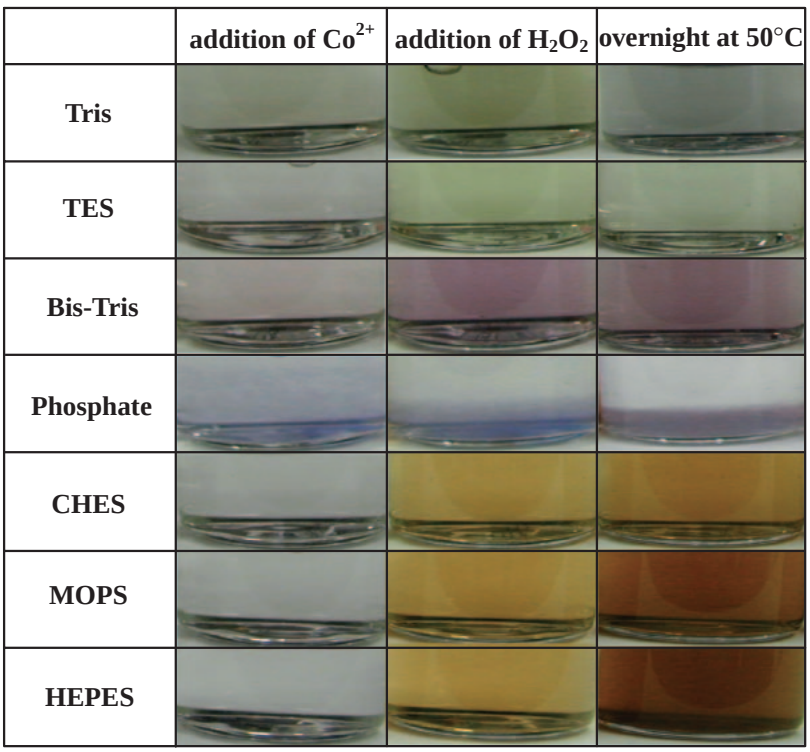
Results and Discussion

Buffer Agent Survey. There are many variables for the aqueous chemical reaction of Co oxide NPs synthesis. It was pointed out that buffer agents sometimes react with metal ions and hinder the designed chemical reaction or metal oxidation.[18] Therefore, we first surveyed the appropriate buffers for the Co oxide core synthesis. Preliminary experiments in which an ACS-H_2O_2 solution was titrated with NaOH resulted in the production of insoluble Co complex precipitates above pH 8.3. Therefore, a solution of several mM ACS with or without 0.5 mg/mL HsAFr in one of the 100 mM Tris, Bis-Tris

Table 1. Color Change Profile of Reaction Mixture[a)]

Buffer	pH	Color change[c)] Addition Co(II)	Addition H_2O_2	Overnight	Core formation[d)]
Tris (7.2–9.4)[b)]	8.3	LG	G	G	N
TES (6.8–8.2)[b)]	8.3	LP	LG	LG	N
Bis-Tris (5.7–7.3)[b)]	8.3	TPu	DPu	Dpu	N
Phosphate (5.8–8.0)[b)]	8.3	↓[e)]	↓[e)]	↓[e)]	N
CHES (8.7–10.3)[b)]	8.3	LP	TB	DB	Y
MOPS (6.5–7.9)[b)]	8.3	LP	TB	DB	Y
HEPES (6.8–8.2)[b)]	8.3	LP	TB	DB	Y

a) Reaction conditions; ferritin (0.5 mg/mL) was prepared in buffer reagent (100 mM). To the aqueous solution of ferritin was ammonium cobalt sulfate (3 mM) was added, followed by diluted hydrogen peroxide (1.5 mM). b) Useful pH range for Good's buffer. c) LG = light green, G = green, LP = light pink, TPu = Thin purple, DPu = dark purple, TB = thin brown, DB = dark brown. d) Y = yes, N = no. e) Insoluble solid was precipitated.



(bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane), TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), phosphate, CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid), MOPS (3-(*N*-morpholino)propanesulfonic acid), and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffers at pH 8.3 was prepared. Then, H_2O_2 at half ACS concentration was added to the solution and the temperature was kept at 50 °C. The bulk precipitates and color change were observed. The results are summarized in Table 1.

In the cases of CHES, MOPS, and HEPES buffers, the addition of H_2O_2 changed the solution color from light pink to thin brown, which is common for these buffer reagents. The color change took about one hour, after which a very slow bulk precipitation began. After being left overnight, the solution had an upper clear brown solution and the very thin layer of precipitates. The UV–visible absorption spectrum of the supernatant revealed a peak around 350 nm, which was used to monitor the cobalt oxide core formation.[14,15] TEM observation with aurothioglucose (2.5%) staining confirmed that cores were formed in some apoferritin cavities. This result indicated that the Co oxide can be synthesized using these buffer agents.

In the cases of Tris, TES, and Bis-Tris buffers, the solution mixture changed color upon the Co(II) addition, which is different from the previous three buffer agents. The color further changed after the addition of H_2O_2 and overnight incubation. There were no bulk precipitates and the solution remained a clear color even after several weeks. Apoferritins in these solutions were observed by TEM with aurothioglucose (2.5%) staining. Only hollow apoferritin molecules were seen and no apoferritin accommodated any cores. No core formation

and no bulk precipitation suggested that all the cobalt ions remained in the reaction mixture solution. The Tris, TES, and Bis-Tris reagents may coordinate Co(III) ions, and form a very stable Co(III) complex ion. Apoferritins in these solutions did not accommodate any cores even after a three-month storage.

In the case of phosphate buffer, the situation is different from the preceding buffers. The solution became turbid upon mixing with apoferritin, Co(II) ion, and phosphate buffer. After oxidization by H_2O_2, a bluish-purple soft precipitation was observed. To investigate this precipitate, we carried out the same experiment but without ferritin molecules. The result was the same, which suggested that cobalt ion and phosphate buffer agent react and produce soft precipitates. Hence, it was concluded that the phosphate buffer is not good for the biomineralization of Co oxide.

Judging from these experimental results, we concluded that CHES, MOPS, and HEPES agents can be used as reaction buffers for oxidizing Co(II) ion and synthesizing Co oxide cores in the apoferritin cavity. On the other hand, some buffer reagents strongly react with the cobalt ions and hinder the biomineralization. This will be true for other metal ion cases. Therefore, a screening test of the buffer agents should be done before every start of experiments on new metal ion biomineralizations. In this study, we selected HEPES buffer for the latter experiments, taking the pH range of the experiments into consideration.

The Best Conditions for Spatially Selective Synthesis of Co Oxide NPs within the HsAFr. Prior reports[14,15] showed that Co(II) ion can be oxidized using H_2O_2, and Co oxide/hydroxide cores can be formed in apoferritin, in which the solution was kept around pH 8.5 by dynamically adding NaOH. In the previous section, we experimentally found that some buffer agents can be used for Co oxide/hydroxide core formation. Therefore, we employed a buffer agent to form Co oxide/hydroxide cores, which is preferable from a mass-production point of view.

Using HEPES buffer, we surveyed the optimum condition for the cobalt core synthesis. We first studied the critical pH above which the H_2O_2 addition produces an insoluble Co complex. The solution of 3 mM Co(II) ion and 37.5 mM Na_2SO_4 was prepared and the pH of this solution was controlled by 100 mM HEPES. To this mixture solution, 1.5 mM H_2O_2 was added and the temperature was kept at 50 °C to accelerate the Co oxide synthesis. After an overnight stand, the solutions above pH 8.0 produced the Co complex precipitates, therefore, the critical pH was revealed to be pH 8.0.

Based on this result, the optimum solution conditions were surveyed. First, we adopted 0.5 mg/mL or approximately 1 μM HsAFr and 37.5 mM Na_2SO_4 as the basic solution. The parameters for the optimum solution survey were the concentration of the Co ion and the solution pH. The H_2O_2 concentration was fixed at half of the cobalt ion concentration which is theoretically enough to oxidize all the Co(II) ions. Since several thousand cobalt ions should be accommodated by one apoferritin molecule, the Co ion concentration range of the survey was determined to be from 1 to 5 mM. The pH range was determined to be from 7.3 to 8.8 taking account of the critical pH. All solutions in the parameter survey matrix were carefully prepared with a total volume of 3 mL and were allowed to stand overnight at 50 °C in air-tight glass bottles.

The results had the following characteristics. When the Co ion concentration is higher than 4 mM, the excessive cobalt ion caused aggregation and made the apoferritin co-precipitate. Few apoferritin molecules were found in the upper solution and ferritin molecules could not be retrieved from the precipitates. On the other hand, in the case of less than a 2 mM Co(II) ion concentration, all the apoferritin cavities cannot be filled. Therefore, there is an optimum Co(II) ion concentration. A similar tendency exists for the solution pH. A higher pH will lead to the fast bulk precipitation and many apoferritin molecules co-precipitate. On the other hand, the lower pH make the cobalt ion very stable and hard to oxidize. Therefore, there is also an optimum pH. Taking these results into consideration, we conducted a detailed survey in the range from 2 to 4 mM cobalt ion concentration and from pH 8.0 to pH 8.6.

Figure 2 shows pictures of the reaction mixtures after an overnight stand in which the difference in the solution state can be visibly seen. The percentage of the ferritin molecules, which formed the core in the cavity over all the ferritin molecules in the solution (CFR), is noted under each picture. The dash represents the fact that there is no ferritin molecule in the solution.

For the 2 mM Co concentration, all the solutions were transparent and the CFR never exceeded 33%. When the solution contained 4 mM cobalt ions, a bulk precipitation was always observed. Considered together with the fact that the precipitates are aggregates of ferritin molecules and Co oxide material, this gallery of pictures suggests that 0.5 mg/mL apoferritin can accommodate Co ions up to around 3 mM, which is the appropriate cobalt ion concentration.

For the 3 mM Co concentration, the solution state drastically changed between pH 8.3 and 8.4. The solution color got darker up to pH 8.3 and a very thin color appeared above pH 8.4. The solution produced little precipitation up to pH 8.3, and the TEM observation showed that the CFR reached 94% at pH 8.3. On the other hand, the solution had a heavy precipitation above pH 8.4 and few ferritin molecules were found in the solution, even the CFR remained high. This indicates that the number of ferritins with the core had a maximum peak at around pH 8.3. This situation becomes clearer by calculating the products of the CFR and the protein concentration in the solution, i.e.) the yield of the core formation (YCF). Figure 3 shows the YCF together with the CFR and protein concentration after the core formation. YCF has a sharp peak at pH 8.3.

Therefore, we conclude that the solution with a 3 mM Co concentration at pH 8.3 is the optimum for the Co oxide biomineralization in the HsAFr cavity.

Cobalt Oxide NPs Synthesis in the Recombinant Apoferritins Cavity. The optimum condition for the Co oxide core synthesis using the recombinant apoferritin Fer-8 was also studied by employing the same protocol. The 3 mM Co concentration was found to be the optimum, which is the same as in Fig. 2. Figure 4 shows the dependences of the protein concentration in the solution after core formation, CFR, and YCF on the solution pH. The bulk precipitation was generally lighter and the protein concentrations in the solution after core formation were not less than 0.35 mg/mL. The CFR and the

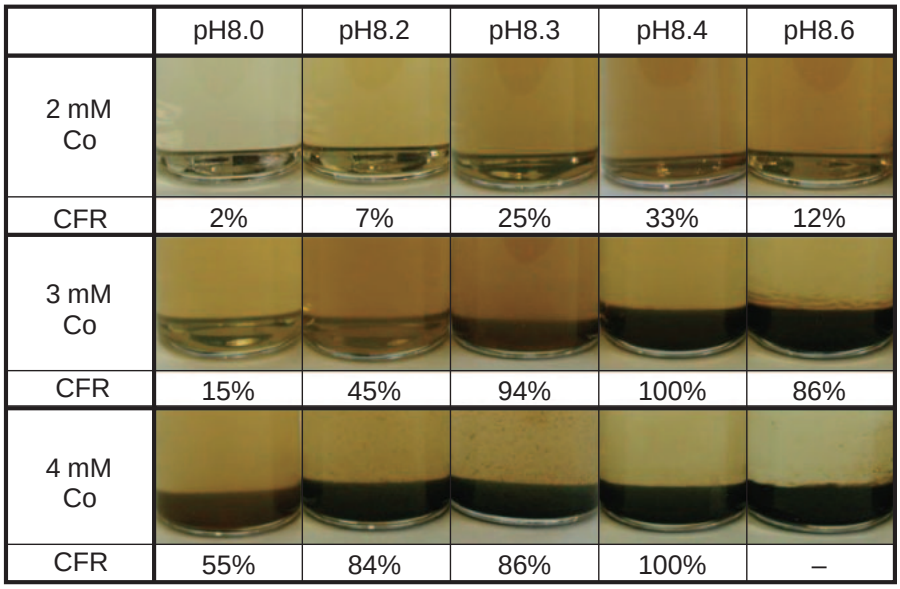


Fig. 2. A gallery of the reaction mixtures of the Co oxide core formation using the HsAFr at 50 °C after overnight stand. The mixture contained 0.5 mg/mL HsAFr, 37.5 mM Na_2SO_4, and H_2O_2 was added with the half concentration of ACS. The clear brown solution contained the ferritin with Co oxide core and the precipitant at the bottom was aggregates of the protein and Co oxides. The core formation ratio, CFR, is written under each picture. The dash implies that there is no ferritin molecule in the solution. The obtained CFR and protein concentration at 3 mM Co ion drastically changed between pH 8.3 and 8.4. The calculated YCF, which is the best parameter for the efficiency of the Co oxide NPs formation, shows a sharp peak at pH 8.3.

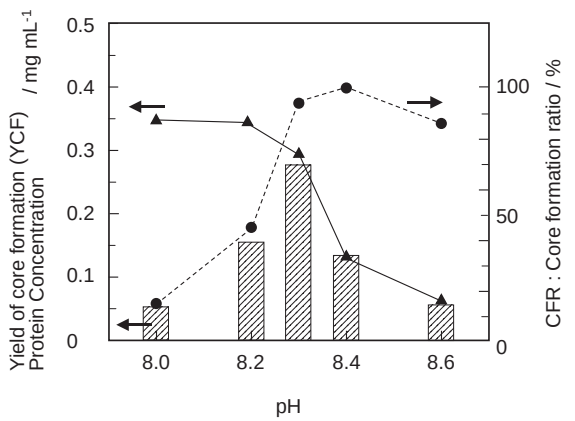


Fig. 3. The dependence of the protein concentration after core formation, CFR and YCF on the reaction mixture pH for the HsAFr. The solution with 0.5 mg/mL HsAFr, 3 mM ACS, 37.5 mM Na_2SO_4, and 1.5 mM H_2O_2 was prepared and its pH was controlled by 100 mM HEPES. The filled triangles denote the protein concentration in the solution after the core formation and the filled circles show the CFR. YCF, which corresponds to the practical protein concentration of the HsAFr with cores, are shown as a bar graph. YCF has a sharp peak at pH 8.3.

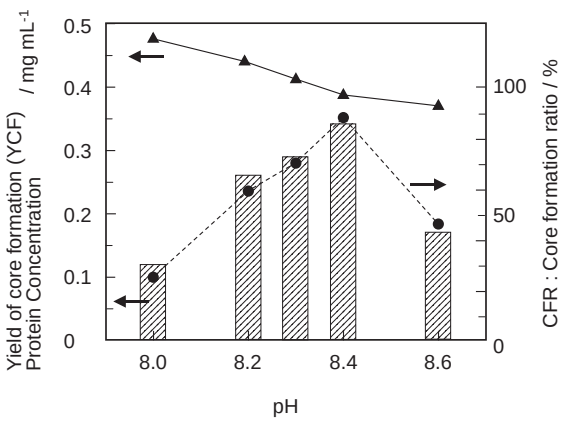


Fig. 4. The dependence of the protein concentration after core formation, CFR and YCF on the reaction mixture pH for the Fer-8. The solution with 0.5 mg/mL Fer-8, 3 mM ACS, 37.5 mM Na_2SO_4, and 1.5 mM H_2O_2 was prepared and its pH was controlled by 100 mM HEPES. The filled triangles show the protein concentration in the solution after the core formation and filled circles show the CFR. YCF, which corresponds to the practical protein concentration of the HsAFr with cores, are shown as a bar graph. YCF has a peak at pH 8.4.

YCF show peaks at pH 8.4, which was slightly higher than the HsAFr case. Based on these results, the optimum conditions for the Co oxide core formation in the Fer-8 cavity are 3 mM Co concentration and at pH 8.4, which are almost the same as those for the HsAFr, but the YCF was significantly improved.

It is clear that the improvement of YCF is due to the difference between HsAFr and Fer-8. HsAFr is mainly composed of L-subunits (90%). The first N-terminus five amino acid residues of the L-subunit in recombinant L-apoferritin, which is composed of only L-subunits, extend out from the protein shell. Therefore, we presume that L-subunit in the HsAFr has such mobile peptides. In comparison, Fer-8 has no such N-terminus peptide. Therefore, this mobile part may have accelerat-

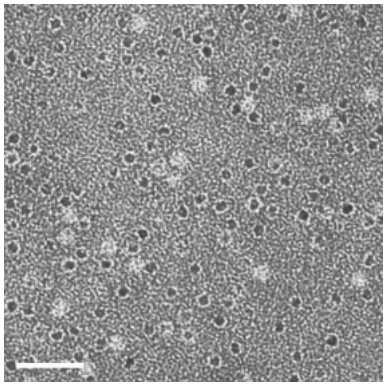


Fig. 5. A TEM image of Fer-8 with Co oxide core stained by aurothioglucose. The Co oxide cores were synthesized using the best conditions described in the results and discussion. They were surrounded by the negatively stained protein shell as clearly seen. The negative staining was done using aurothioglucose, which cannot penetrate into the apoferritin cavity and cannot stain the inner cavity. The scale-bar is 50 nm.

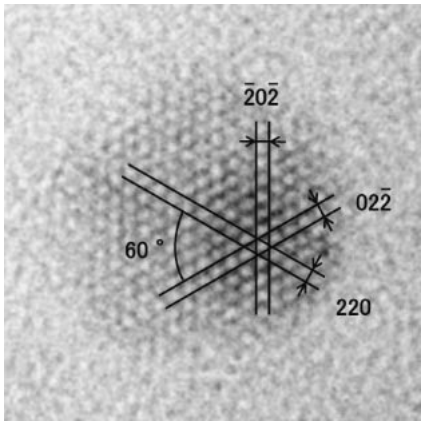


Fig. 6. The high resolution TEM image of the synthesized Co oxide core without staining. The lattice image of one of the synthesized Co oxide cores is clearly seen. The lattice lines cross each other at an angle of 60° and the lattice distance is 0.284 nm on average, which indicates that the core structure is Co_3O_4 and the lattice is the (220) plane.

ed the Co oxide nucleation in the bulk solution and caused more bulk precipitates, or the extended residues may have hindered the incorporation of Co ion into the cavity. Another possibility is that H-subunit which composes 10% of the protein shell may have affected the core formation.

Characterization of the Co Oxide Core Synthesized in the Apoferritin Cavity. The synthesized cobalt oxide was characterized by X-ray photoemission spectroscopy (XPS) measurements in order to confirm the existence of cobalt atoms in the core. The main peak at 788.5 eV for Co 3d was clearly observed.

Secondly, high resolution TEM observations and EELS measurements were carried out using cores synthesized by Fer-8. Figure 5 shows the TEM image of the Fer-8 with Co oxide cores stained with aurothioglucose. The Co oxide cores were surrounded by a negatively stained protein shell. Almost all the apoferritins accommodate cores and their sizes looked homogeneous. An EELS measurement was carried out using the same sample but without staining and the spectra showed the existence of cobalt in the cores, which was consistent with the XPS result. The XPS and EELS measurements confirmed that the cores included cobalt atoms.

A high resolution TEM image of the core synthesized at 50 °C without staining is shown in Fig. 6. The lattice image is clearly seen. The angles between the lattices are exactly 60 degrees and the measured lattice distances ranged from 0.281 to 0.288 nm. These data indicate that these lattices correspond to the Co_3O_4 (220) planes. Considered together with the XPS and EELS observations, these results suggest that the structure of the cobalt core is Co_3O_4.

Third, X-ray powder diffraction (XRD) was carried out. Figure 7 shows the typical diffraction pattern from the Fer-8 cores. The peaks were fairly broad, but it was possible for peak positions to be determined. The set of diffraction peaks match those of the Co_3O_4 crystal, which is consistent with the previous HR-TEM lattice observation. The average single crystal size of the cores was calculated from the half-width of the diffraction peaks using Shearer's equation and it was 6 nm. As the Fer-8 cavity size is about 7 nm, this result indicated that the cores are nearly single crystals. On the other hand, in the case of cores formed at 30 °C, the average size of single crystal domains was calculated to be less than 3 nm (data not shown). The Co oxide NP formation is thought to be composed of two steps. The first step is that the small nuclei formed at the nucleation sites of the apoferritin cavity surface and the second step is the self-catalytic crystal growth. Therefore, the polycrystalline Co_3O_4 NPs may be due to the multi Co_3O_4 nucleation at some of the nucleation sites. The elevated synthesis temperature may lead to the fast crystal growth which suppresses the multi nucleation and promotes the single Co_3O_4 crystals.

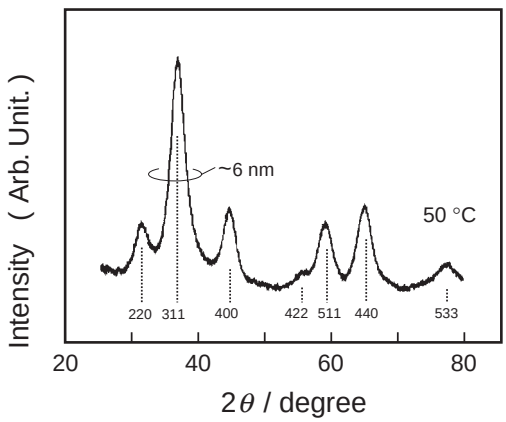


Fig. 7. A XRD pattern from synthesized Co oxide cores in the Fer-8 cavity. The positions and heights of broken lines represent XRD peak-positions (2θ) and diffraction-intensities from a cubic Co_3O_4 crystal, respectively (JCPDS card PDF#43-1003). This result indicates that the crystalline material in the core is Co_3O_4. From the half-width of the largest diffraction peak, the average crystal size is calculated to be 6 nm, which is nearly the same size as the protein cavity.

Conclusion

We have shown that Co_3O_4 NP with the diameter of 6 nm can be efficiently synthesized in the HsAFr and Fer-8 cavity. A thorough survey revealed the optimum conditions for the Co_3O_4 NP synthesis to be: 0.5 mg/mL apoferritin, 37.5 mM Na_2SO_4, 100 mM HEPES, pH 8.3, 3 mM ACS, and 1.5 mM H_2O_2 at a temperature of 50 °C. Under these conditions, almost all the apoferritins contain Co_3O_4 NPs. It also became clear that the selection of the buffer reagent is critical to the Co oxide biomineralization. Some buffer reagents strongly react with cobalt ions and hinder the biomineralization. Therefore, screening of buffer reagents should be done before the start of every experiment for new metal ion biomineralizations. The synthesized Co oxide cores are confirmed to be Co_3O_4 by XPS, EELS, XRD, and high resolution TEM. The knowledge obtained in this study relating to Co_3O_4 NP synthesis inside apoferritin will provide valuable information for the biomineralization of other inorganic materials using bio-templates. Since the cobalt oxide NPs can be reduced easily to metallic cobalt by several methods, including heat treatment under reductive gas, we are now studying the feasibility of fabricating a memory device using synthesized Co oxide NPs.

The authors thank Mr. T. Hikono for XPS measurements and Mr. M. Ueda for XRD measurements.

References

1 W. H. Massover, *Micron*, **24**, 389 (1993).
2 P. M. Harrison, S. C. Andrews, P. J. Artymiuk, G. C. Ford, J. R. Guest, J. Hirzmann, D. M. Lawson, J. C. Livingstone, J. M. A. Smith, A. Treffry, and S. J. Yewdall, *Adv. Inorg. Chem.*, **36**, 449 (1991).
3 T. Furuno, H. Sasabe, and K. M. Ulmer, *Thin Solid Films*, **180**, 23 (1989).
4 H. Yoshimura, T. Scheybani, W. Baumeister, and K. Nagayama, *Langmuir*, **10**, 3290 (1994).
5 I. Yamashita, *Thin Solid Films*, **393**, 12 (2001).
6 D. Price and J. G. Joshi, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 3116 (1982).
7 J. Fleming and J. G. Joshi, *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7866 (1987).
8 F. C. Meldrum, V. J. Wade, D. L. Nimmo, B. R. Heywood, and S. Mann, *Nature*, **349**, 684 (1991).
9 F. C. Meldrum, B. R. Heywood, and S. Mann, *Science*, **257**, 522 (1992).
10 J. F. Hainfeld, *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 11064 (1992).
11 F. C. Meldrum, T. Douglas, S. Lei, P. S. Arosio, and S. Mann, *J. Inorg. Biochem.*, **58**, 59 (1995).
12 T. Douglas, D. P. E. Dickson, S. Betteridge, J. Charnock, C. D. Garner, and S. Mann, *Science*, **269**, 54 (1995).
13 K. K. W. Wong and S. Mann, *Adv. Mater.*, **8**, 928 (1996).
14 T. Douglas and V. T. Stark, *Inorg. Chem.*, **39**, 1828 (2000).
15 M. Allen, D. Willits, M. Young, and T. Douglas, *Inorg. Chem.*, **42**, 6300 (2003).
16 M. Okuda, K. Iwahori, I. Yamashita, and H. Yoshimura, *Biotechnol. Bioeng.*, **84**, 187 (2003).
17 I. Yamashita, J. Hayashi, and M. Hara, *Chem. Lett.*, **33**, 1158 (2004).
18 E. A. Lance, C. W. Rhodes, III, and R. Nakon, *Anal. Biochem.*, **133**, 492 (1983).