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Estimation for diameter of superparamagnetic particles in *Daphnia* resting eggs

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Ferromagnetic resonance (FMR) with an electron spin resonance (ESR) apparatus was investigated for superparamagnetic particles within *Daphnia* resting eggs. High-field (HF) resonance lines near g=2 resulted from single superparamagnetic particles, were detected from ESR spectra of *Daphnia* resting eggs. The size of isolated superparamagnetic particles within *Daphnia* resting eggs was calculated to be approximately 13 nm in diameter by analysis of the temperature dependence of the HF line width. Small-angle X-ray scattering (SAXS) analysis of *Daphnia* resting eggs also showed that average size of superparamagnetic particles in diameter, equivalent to magnetite, was approximately 13 nm. The combination of FMR and SAXS measurement is very effective in estimating the size of superparamagnetic particles in biological organisms, with difficulties of preparing for samples for measurement by electron microscopy. However, *Chlorella*, with that *Daphnia* were raised, did not show FMR spectra, showing no magnetic particles within *Daphnia* resting eggs. Therefore, it suggested that superparamagnetic particles within *Daphnia* resting eggs, were mineralized in *Daphnia* as the result of biomineralization of Fe originated from *Chlorella*.

Key words: *Daphnia* resting egg, magnetite, superparamagnetic particle, FMR, SAXS

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Daphnia can switch reproduction way from parthenogenesis to sexual reproduction under unfavorable environments, resulting from producing robust resting eggs[1,2]. *Daphnia* resting eggs are known to have some unique adaptive and survival abilities. For example, resting eggs can remain viable for decades, and can withstand freezing and drying[3]. Resting eggs can also survive in the harsh environment of a predator's digestive system[4], eventually being excreted intact. In addition to these characteristics, a previous study using a nondestructive method to measure static magnetic moments showed that ferromagnetic materials, probably magnetite Fe_3O_4, exist in *Daphnia* resting eggs[5]. From two types of analysis, the Moskowitz test[6–9] and the comparison of temperature dependence of magnetization after zero-field cooling and field cooling, we also proposed that some part of the magnetic ions, Fe^{3+} and Fe^{2+}, in *Daphnia* resting eggs exist in superparamagnetic particles[10]. However, the size of such particles contained in *Daphnia* resting eggs was remained unknown.

Magnetic resonance is a nondestructive method to give important information of the state of magnetic ions in samples. Particularly, ferromagnetic resonance (FMR) leads to estimation of diameter of superparamagnetic particles[7–9]. Superparamagnetic particles of magnetite contained in ants have been directly characterized using FMR[11], and their diameter was calculated to be ~13 nm. Small-angle X-ray scattering (SAXS) is another nondestructive method used in material analysis to study average diameter of particle and its distribution contained in a sample. Thus, the combination of FMR and SAXS measurement is very effective in estimating the size of superparamagnetic particles in complex biological organisms, with difficulties of preparing for samples for measurement by electron microscopy.

The purpose of the current study is (1) to nondestructively estimate and measure the diameter of superparamagnetic particles within *Daphnia* resting eggs by FMR and SAXS, and (2) to investigate magnetic characteristics of *Chlorella* with that *Daphnia* were raised. The current study demonstrates that the average diameter of superparamagnetic particles in *Daphnia* resting eggs was estimated to be approximately 13 nm from both FRM and SAXS results. The ESR signals from superparamagnetic particles were not detected in *Chlorella*. Superparamagnetic particles within *Daphnia* resting eggs are probably bio-mineralized in *Daphnia* itself.

Materials and Methods

Daphnia magna

Daphnia magna were raised based on OECD criteria[12] with *Chlorella vulgaris*, maintained at 20 ± 2°C and exposed to 16-hour period of light followed by an 8-hour period of darkness per day. 50 ml of *Chlorella* (*Chlorella vulgaris Chikugo*, Chlorella Co., Tokyo) was centrifuged for 15 min at 3,000 rpm. After centrifugation, the pellet was dissolved in ultra pure water and then centrifuged again for 15 min at 3,000 rpm. These procedures were repeated two more times. Finally, the suspension was diluted up to 7.5×10^8 cells/ml and was used to culture *Daphnia*. To obtain resting eggs, 60 three- to four-week-old *Daphnia* were cultured in one liter of medium. When the population density approached the overcrowding level of 480 per liter, the *Daphnia* began to produce resting eggs, which were collected, rinsed with de-ionized water, and dried before measurements. All measurements including handling *Daphnia* were conducted with non-magnetic instruments.

Ferromagnetic resonance (FMR)

Ferromagnetic resonance (FMR) measurement was done using a conventional electron spin resonance (ESR) spectrometer (JES-TE200, JEOL, Tokyo) operating at an X-band frequency (9.4 GHz) at several temperatures between 24 K and 315 K. Reference signals of Mn^{2+} in MgO were used as the standard for g-values.

Small-angle X-ray scattering (SAXS)

Small-angle X-ray scattering (SAXS) for *Daphnia* resting eggs were measured by an X-ray diffractmeter (Rint-TTR III, Rigaku Co., Japan) operated at 50 kV/300 mA using CuKα radiation (1.5418 Å). The range of measurement was 0.1°–8.0° on 2θ scale, and the step was 0.02°. The distribution of the diameter of particle was analyzed by NANO-Solver® (Rigaku Co., Japan).

Results and Discussion

Figure 1 shows electron spin resonance (ESR) spectra of dried *Daphnia* resting eggs measured at several temperatures between 24 K and 315 K. Each spectrum has a few characteristic structures: (1) a resonance line detected at g = 4.3, (2) a sharp line signal at g = 2.0, and (3) a pair of narrow and broad lines overlapped with each other around g = 2. They will be called as "signal A", "signal B", and "signal C", respectively, in the following discussions.

Wajnberg *et al*. and El-Jaick *et al*. reported the understanding of ESR spectra in ant *Pachycondyla marginata*[11] and honeybee *Apis mellifera*[13]. Wajnberg attributed the similar line of signal A to isolated iron ions in grassy matrix[14], and the similar line of signal B to free radicals resulting from biological processes[15], respectively. The similar line of signal C was also detected in ants[11]. According to their interpretation, the similar line of signal C is ferromagnetic resonance (FMR) due to nanoparticles of a ferromagnet substance. Such particles are also the origin of superparamagnetic behavior in static magnetic measurement. Wajnberg reported that the similar line of signal C was divided with Gaussian function into two lines, a narrow line and a broad line. Wajnberg defined a broad line as a high field (HF) resonance line[11]. HF line is characteristics of FMR, originated from single magnetic nanoparticles, and its intensity increased and the HF width narrowed slightly, with temperature increasing[11,16–18].

A similar analysis of the spectra by Gaussian fitting was performed for interpreting the signal C of *Daphnia* resting eggs. Figure 2 shows the result of Gaussian fitting of the signal C at 88 K divided into a narrow (C_1) line and a broad (C_2) line. The line shape around g = 2 in Figure 2 is well reproduced by the sum of two Gaussian curves of C_1 line and C_2 line. C_2 were divided from ESR spectra at all measurement temperatures, showing increasing its intensity and narrowing its line width with temperature increasing. This C_2 behavior is characteristic of FMR. Therefore, C_2 is

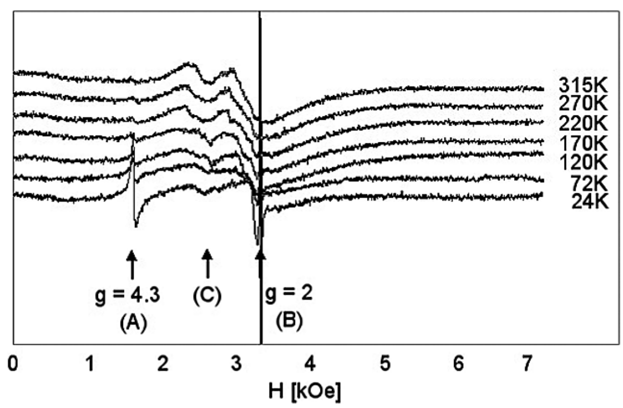

Figure 1 ESR spectra on *Daphnia* resting eggs measured at several temperatures between 24 K and 315 K.

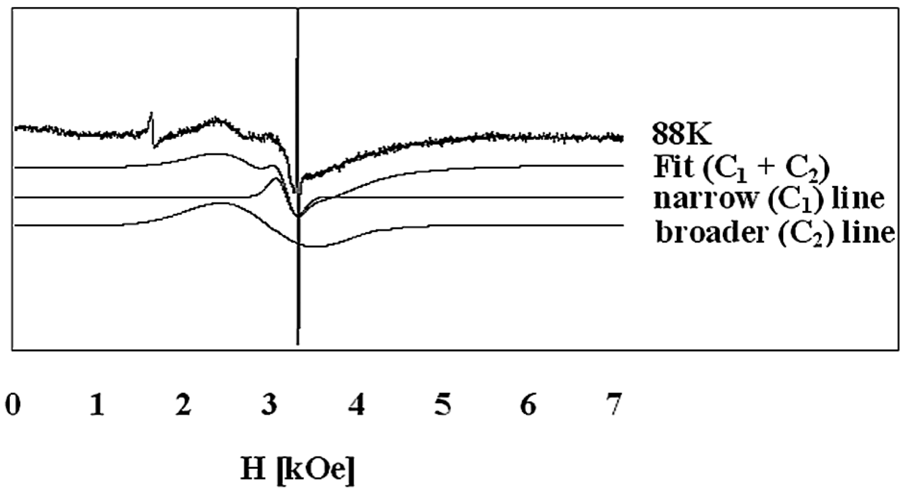

Figure 2 ESR spectra on *Daphnia* resting eggs measured at 88 K. ESR spectra of *Daphnia* resting eggs at 88K divided into narrow (C_1) line and broader (C_2) line with Gaussian function. A combined line with narrow and broad components, showing a good fitting with an experimental result.

equivalent to HF line.

Wajnberg reported the narrow line around g = 2 of ESR spectra of ant showed the increased intensity with increasing temperature[11]. Thus, the contribution of a nanoparticle precursor to this narrow line can also be considered. Contrary to this result, C_1 of ESR spectra of *Daphnia* resting eggs showed the decreased intensity with temperature increasing. Therefore, it suggested that if precursors of superparamagnetic particles exist in the eggs, the characteristics of these precursors are different form those of ants. Further studies are needed to know this point in near future.

Wajnberg also reported that a broad line divided from ESR spectra in the low field with Gaussian function, defined as a low field (LF) resonance line. LF line is another characteristics of FMR, originated from a chain or aggregate of single magnetic nanoparticles[11,16–18]. Although a broad line, equivalent to LF line, in the low field was detected in the ESR spectra on *Daphnia* resting eggs, the ESR spectra of all temperature measurement could not be divided into LF with Gaussian function in this study. Therefore, the existence of a chain or aggregate of single magnetic particles in *Daphnia* resting eggs must be considered in the future study.

Figure 3 shows temperature dependence of the resonance line width ΔH_R of the HF line of *Daphnia* resting eggs. The line width decreased abruptly above about 130 K. Similar behavior of HF line width was reported in ant[11]. According to Morais *et al.*, the temperature dependence of the line width of ESR of superparamagnetic particles dispersed in non-magnetic matrix is expressed by

$$\Delta H_R = \Delta H^0_R \tanh (\Delta E/2k_B T), \qquad (1)^{19}$$

where ΔE is the energy barrier for a magnetic moment of a particle to switch from one direction to another. If the particle is nearly a sphere, a cube, or other shape of high symmetry,

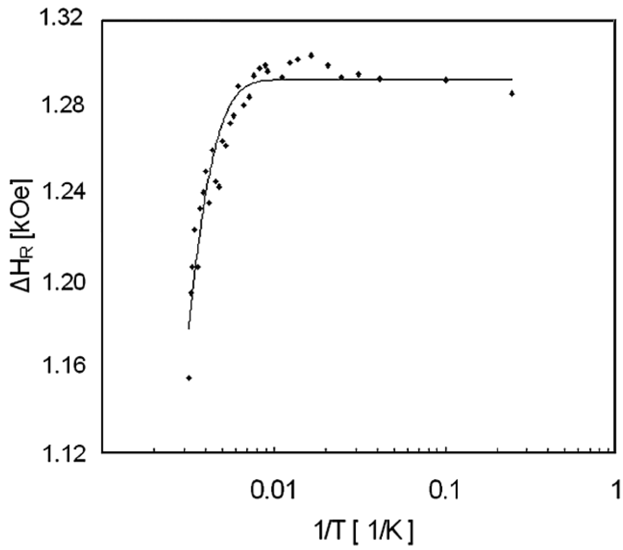

Figure 3 Temperature dependence of the resonance line width of the HF resonance line.

it is given by

$$\Delta E = a|K|V, \qquad (2)$$

where K and V are a magnetocrystalline anisotropy constant and the average volume of particles, respectively. A numerical factor a is a constant of the order of magnitude of 1, depending on the switching process. The width at the high temperature limit ΔH^0_R is expressed as

$$\Delta H^0_R = 5g\beta Sn/D^3, \qquad (3)^{19}$$

where g = electron g-factor, β = the Bohr magneton, S = the spin number associated with a magnetic ion, n = the number of magnetic ions in a particle, and D = the average particle-particle distance. Non-linear least squares fitting of the relation (1) to the results in Figure 3 gives the result that

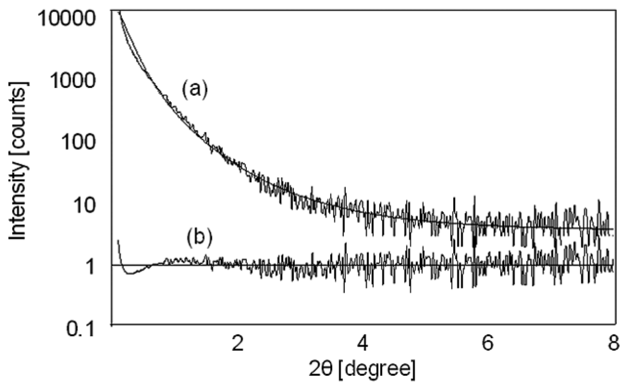

Figure 4 SAXS profile for the *Daphnia* resting egg: (a) scattering profile and fitting curve; (b) residue.

$\Delta H^0_R = 1292$ Oe and $\Delta E/2k_B = 483$ K.

If we assume that $a = 1/3$, $|K| = 2 \times 10^4$ J/m^3, and $S = 5/2$ (the spin number of an Fe^{3+} ion), considering that the small particles are composed of Fe_3O_4, we obtain the following results: $V = 2 \times 10^{-24}$ m^3, which means that the average diameter r is approximately 13 nm, and $D = 2$ μm. Although the anisotropy constant K depends on temperature within the range of our measurement, changing from 1 to 4×10^4 J/m^3 in the temperature range of our measurement, the typical volume in magnetite, 2×10^4 J/m^3, was used in this assumption. The above estimation values of r and D should be taken as showing roughly the order of magnitude of r and D.

Some points under 130 K region seems deviated from the fitting curve in Figure 3. Wajnberg reported the similar phenomenon resulting from aggregated magnetic nanoparticles not from isolated nanoparticles in ants[11]. Therefore, it seems that aggregated nanoparticles of magnetite possibly exist in *Daphnia* resting eggs. However, LF, originated from a chain or aggregated magnetic nanoparticles, were not detected by Gaussian fitting in Figure 2. More studies are needed to understand this in the near future.

Figure 4 shows the SAXS scattering an intensity profile for *Daphnia* resting eggs and the results of curve fitting, on an assumption of small magnetite particles. Close fitting was seen in the region around two degrees for two theta in Figure 4, resulting from small deviation of SAXS measurement data with low angle of scattering X-ray. The results are summarized as the diameter-distribution curve in Figure 5. The average diameter of particles was estimated to be 13 nm, which is very near to the value from FMR study. Fitting curve in the very low angle in Figure 4 shows that there is a possibility of existing bigger particles in *Daphnia* resting eggs. More studies are needed to understand this in the near future.

Figure 6 shows ESR spectra of dried *Chlorella* at 300 K, being quite different from the ESR spectra of *Daphnia* resting eggs. It is composed of a sharp single line and a set of six lines around g = 2. They are attributed to free radicals resulting from biological processes like the signal B in

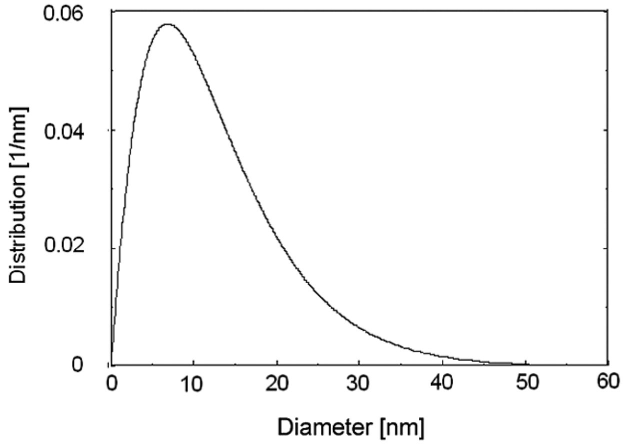

Figure 5 Diameter distribution of superparamagnetic nanoparticles within *Daphnia* resting eggs.

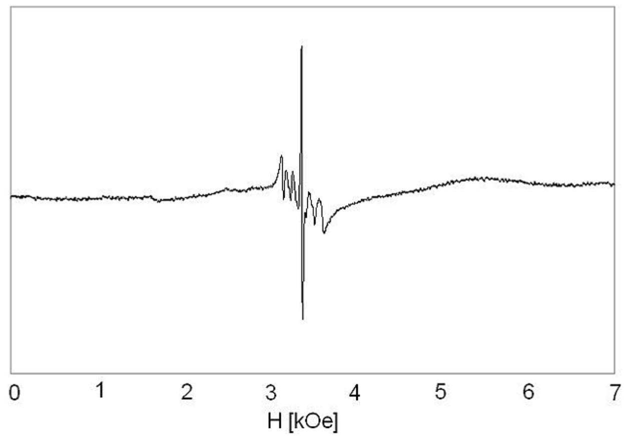

Figure 6 ESR spectra on *Chlorella* measured at 300 K.

Figure 1, and to Mn^{2+} like those observed in the samples of plants leaves[20,21]. As the result of analysis by an inductively coupled plasma — mass spectrometry (ICP-MS), *Chlorella*, with that *Daphnia* were raised, contained Fe as 170 ± 10 mg/kg. Therefore, it suggested that superparamagnetic particles within *Daphnia* resting eggs, were mineralized in *Daphnia* as the result of biomineralization of Fe originated from *Chlorella*.

Dried *Daphnia* resting eggs were used in this study to avoid noise from water in the samples. However, a denaturation of Fe_3O_4 to Fe_2O_3 could be occurred by the process of drying. Therefore, a total amount of superparamagnetic particles in the samples could be reduced compared with those contained in intact *Daphnia* resting eggs.

Other materials except magnetite, which may affect ferromagnetic and superparamagnetic resonance, were considered as follows. Ferritin, an iron-storage protein, is known to accumulate iron ions into its core as hydrous ferric oxide crystallites of various size up to 8 nm in diameter surrounded by a shell of protein of molecular weight 450,000 Da, capable of storing up to 4,500 iron atoms[22]. Since iron exist as ferric oxide in the ferritin core, ferritin tends to be considered as magnetic material itself. However, it seems that ferritin does not show magnetism in nature, because ferric oxide exists as "hydrous" condition. Moreover, the resting eggs were performed to ESR after air-dried at room temperature with no artificial heating in the current study. Therefore it is hard to consider that ferritin directly affects the ferromagnetic and superparamagnetic resonance pattern. Wajnberg *et al.* has reported a temperature behavior of ferritin ESR spectra, showing an existence of moved resonance line from $g = 9$ to $g = 4.3$ with temperature increase from 80 K to 290 K[22]. The current ESR spectra of the resting eggs did not show the phenomenon that g value shifted greatly. Therefore, the current data show that ferromagnetic and superparamagnetic resonance resulted from magnetic materials, and not from ferritin.

Weiss *et al.* reported that there are two types of process of producing magnetic materials in bacteria[7]. Magnetotactic bacteria produce magnetite by a "genetically controlled" process. In contrast, dissimilatory iron-reducing bacteria typically form magnetite extracellularly as a consequence of iron reduction for energy generation, a "biologically induced" process. If a process of producing superparamagnetic particles in *Daphnia* resting eggs are cleared whether "genetically controlled" or "biologically induced", it will lead us to better understand the physiological meaning why superparamagnetic particles exist in *Daphnia* resting eggs.

In conclusion, the current study revealed that superparamagnetic particles of the average diameter of approximately 13 nm are present in *Daphnia* resting eggs. They are separated from each other by the average distance of a few hundreds times of particle diameter itself. The combination of FMR and SAXS is very effective non-destructive methods in estimating the diameter of superparamagnetic particles in other complex biological organisms, with difficulties of preparing for samples for measurement by electron microscopy.

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