

SUPERPARAMAGNETIC MAGNETITE IN THE IRON GRANULES OF HONEYBEES (*APIS MELLIFERA*)

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Abstract

Magnetoreception and iron biomineralization in honeybees (*Apis mellifera*) have been investigated for several decades. Numerous open questions are still been hotly debated, especially the location of superparamagnetic magnetite. To solve this argument, we use the size-density purification technique to purify the iron granules of trophocytes and determined their magnetic properties, which included magnetism by superconducting quantum-interference device magnetometers, magnetic domain by atomic force microscope and magnetic force microscope, as well as ionic valence by electron paramagnetic resonance and electron spectroscopy for chemical analysis. Taken together these results show that superparamagnetic magnetite and FeOOH are clearly present in iron granules of trophocytes. This research clearly confirms that superparamagnetic magnetite is present in iron granules, and reasonably establishes a clear link between iron biomineralization and magnetoreception.

Introduction

Magnetoreception of honeybees (*Apis mellifera*) has been demonstrated by behavioral changes, such as in comb building, homing orientation and discriminative choice, as affected by applied magnetic fields (Frier et al., 1996; Gould, 1986; Gould et al., 1980; Kirschvink and Kirschvink, 1991; Kirschvink et al., 1997; Walker et al., 1989; Walker and Bitterman, 1985; 1989a; 1989b). These behavioral studies suggest that biomagnetites (Fe_3O_4) are deposited as magnetoreceptor, which plays a role as a transducer of the magnetic field information. Ferromagnetic magnetite has been suggested to exist at the anterior and dorsal region of the abdomen, based on the results of studies with Curie temperature and magneto-hysteresis loops by a superconducting quantum-interference device (SQUID) from the abdomens of dried dead bees (Gould et al., 1978), as well as based on the result of study with small-magnetized wires glued at various locations on the free-flying bees (Walker and Bitterman, 1989a; 1989b). However, so far, no biologically deposited iron minerals have been found at this suggested region.

Conversely, numerous iron granules (IGs) were found in the trophocytes of the ventral abdomen of honeybees. Initially, IGs were regarded as amorphous granules by regular transmission electron diffraction (Hsu and Li, 1993; Kuterbach et al., 1982; Kuterbach and Walcott, 1986a; 1986b). IGs were confirmed to be magnetic when the crystalline structure of superparamagnetic magnetite (SM), in the size of ~ 10 nm, was found in the center of IGs

under high-resolution transmission electron microscopy (HRTEM) (Hsu and Li, 1994). Subsequently, a hypothesis for magnetoreception of honeybees has been postulated on the basis of the finding of SM of IGs, the attachment of cytoskeletal system on the membrane of iron deposition vesicles and the innervations of neural system on the trophocytes (Hsu and Li, 1994). These findings have reasonably established a clear link between iron biomineralization and magnetoreception. However, this hypothesis was questioned, and IGs were suggested to be the degraded forms of holo-ferritin, not magnetite (Kirschvink and Walker, 1995; Nesson, 1995; Nichol and Locke, 1995).

In order to resolve this argument, it is necessary to purify IGs and obtain a huge amount of IGs to measure the magnetic properties directly. In this study, we utilize the newly developed technique called the size-density purification technique (Hsu, 2006) to purify the IGs of trophocytes and determined their magnetic properties by a variety of magnetite-determining techniques including SQUID, atomic force microscope (AFM), magnetic force microscope (MFM), electron spectroscopy for chemical analysis (ESCA) and electron paramagnetic resonance (EPR). This study will help us to determine whether SM is present in IGs of trophocytes in honeybees.

Results

Numerous IGs from the trophocytes of honeybees were obtained by using the size-density purification technique. These IGs are electron-dense when they were observed

under TEM (Fig. 1A). Most of the membrane of iron deposition vesicles is lost during purification, but some membrane is still present (Fig. 1B). Various magnetic properties of purified IGs were determined, which included magnetism by SQUID magnetometers, magnetic domain by AFM and MFM, as well as ionic valence by EPR and ESCA.

An averaged SQUID magnetization curve of IGs from 12,000 honeybees showed that IGs possess a residual magnetization of 2.5×10^{-5} emu, a saturation magnetization of 2.83×10^{-4} emu, and an intrinsic coercivity of 100-150 Gauss (Fig. 2). Hence, these IGs are magnetically soft. The soft-hysteresis loops reflect the easy axis of magnetization, which is in-plane on the surface, as a result of the magnetite in IGs of trophocytes. The smallness of coercivity confirms that the magnetite exists in a superparamagnetic state.

The image of AFM of an IG from 8,000 honeybees showed that the height and lateral diameter of iron granule is about 60 nm and 1 μm , respectively (Fig. 3A). The image of MFM of same IG showed that the single domain inherent in each iron granule reveals superparamagnetic behavior (Fig. 3B). Ripples appeared tightly when the MFM tip scanned across a strong domain walls, as the scan speed was accelerated and became direction dependent. The AFM and MFM experiments were run in dynamic, non-contact mode under high vacuum conditions to enhance the sensitivity.

The ESCA spectra of IGs from 40,000 honeybees showed that FeO and Fe₂O₃ coexist in IGs (Fig. 4A). The binding energy of Fe(2p_{1/2}) of FeOOH shifted about 715.6 eV and of

Fe(2p_{1/2}) of FeO about 713.2 eV, while carbon (1s) shifted about 3.8 eV (data not show). The shifts of Fe(2p_{3/2}) and Fe(2p_{1/2}) of Fe₂O₃ were about 713.8 eV and 727.5 eV, respectively. These results suggest that the IGs contain Fe₃O₄ and FeOOH. Additionally, the ESCA results indicate that the purified IGs contained oxygen, carbon, nitrogen, iron, phosphorus, silicon and calcium (Fig. 4B).

The EPR spectra from IGs from 80,000 honeybees show that magnetite (Fe₃O₄) existed in IGs (Fig. 5). We attribute the extraordinary broad spectra to the blocked superparamagnetic resonance of the dominative large-grain magnetites, which emits ferromagnetic resonance. The distribution of small nanoparticles is much less than the large one. We surmise that the much sharper, yet weak, signal in the inset occurring at g~ 2.0 is the spin resonance of Fe²⁺ ions. Thus the extremely small peak centered at g~ 4.3 may be the isolated spin ⁶S_{5/2} of the remnant Fe³⁺ ions, as the second-order crystal field coefficient with axial symmetry vanishes while that with rhombic symmetry persists. The spectroscopic results indicate that IGs apparently contain single-domain SM. The above-mentioned results show that SM is clearly present in the IGs of trophocytes in honeybees.

Discussion

In the current study, we used the size-density purification technique to purify numerous IGs from trophocytes, by which we have determined SM and FeOOH in purified IGs by SQUID, AFM/MFM, ESCA and EPR. This result is consistent with the previous result of

HRTEM (Hsu and Li, 1993) and definitely confirms that SM is present in IGs. This study is the first to use a large quantity of IGs to study the magnetic properties of IGs in honeybees. By means of this method, the results are more convincing than that of HRTEM. However, no matter which methods are used, the results are the same. Therefore, SM is clearly present in ion granules of trophocytes in honeybees.

There are many evidences supporting the presence of SM in honeybees, including behavior evidences (Frier et al., 1996; Gould, 1986; Gould et al., 1980; Kirschvink and Kirschvink, 1991; Kirschvink et al., 1997; Walker et al., 1989; Walker and Bitterman, 1985; 1989a; 1989b) and magnetic measurement evidences of the whole body of dead honeybees (El-Jaick et al., 2001; Ferreira et al., 2005; Gould et al., 1978). Biomagnetite was first suggested to exist at the anterior and dorsal region of the abdomen for several decades (Gould et al., 1978; Walker and Bitterman, 1989a; 1989b), but, so far, no biologically deposited iron minerals has been found at this suggested region. In contrast, numerous IGs were found in the trophocytes of the ventral abdomen of honeybees (Hsu and Li, 1993; Kuterbach, et al., 1982; Kuterbach and Walcott, 1986a; 1986b). IGs were originally regarded as amorphous granules and not magnetite containing (Hsu and Li, 1993; Kuterbach, et al., 1982; Kuterbach and Walcott, 1986a; 1986b) until SM was found in the center of IGs under HRTEM (Hsu and Li, 1994). Nevertheless, IGs were still regarded as the degraded forms of holoferritin, not magnetite (Kirschvink and Walker, 1995; Nesson, 1995; Nichol and Locke, 1995). The

location of SM in honeybees is hotly debated (Hsu and Li, 1995). This debate results from the lack of a large quantity of pure IGs to determine their magnetic properties. Thus, we use the size-density purification technique (Hsu, 2006) to purify a huge amount of IGs and study their magnetic properties by numerous magnetic-determining techniques. The results obtained from a huge amount of IGs and numerous magnetic-determining techniques should be convincing. The results of this study confirm that SM is clearly present in IGs and the magnetic moment of the whole body of dead honeybees should derive from the SM of IGs. These results should resolve the debate, reasonably establishes a clear link between iron biomineralization and magnetoreception, and provides solid basis for further study of magnetoreception in honeybees.

The magnetic moment of freshly killed bees and dead bees was first detected at 700 Gauss by SQUID. The average of magnetic moment of bees is about 2.0×10^{-6} emu (Gould, et al., 1978). However, there is no hysteresis curve obtained. Later, a hysteresis curve of naturally dead honeybees was detected by SQUID (Ferreira, et al., 2005). It showed that intrinsic coercivity of honeybees is at the interval of 83-103 Oe. In this study, however, a hysteresis curve of a huge amount of purified IGs was detected by SQUID. The intrinsic coercivity is at the interval of 100-150 Gauss (Oe). Both values of intrinsic coercivity show that honeybees contain SM. However, one was derived from naturally dead honeybees; while the other was derived from a huge amount of purified IGs in trophocytes. So far, IGs are

found only in the trophocytes. Therefore, both magnetic moment should be from the SM in IGs.

AFM and MFM are generally used to study SM particles (Raşa, et al., 2002). They have been used to determine the magnetite in rainbow trout (Diebel et al., 2000). In this study, AFM and MFM were first used to determine the SM in the IGs of honeybees. The results are similar to previous studies (Diebel et al., 2000; Raşa, et al., 2002) and show that SM is present in IGs.

The EDX spectra of numerous purified IGs of ESCA (in this article) are the same as the EDX spectra of an iron granule of STEM (Hsu, 2006; Kuterbach et al., 1982). It showed no other contaminations in a huge amount of purified IGs. The EDX spectral of an iron granule of STEM (Hsu, 2006; Kuterbach et al., 1982) and the EDX spectral of numerous purified IGs of ESCA (in this article) showed the absence of manganese (Mn) signal. In addition, the typical X-band resonance spectra from numerous purified IGs also showed the absence of manganese (Mn) signal (in this article). However, the typical X-band resonance spectra from the abdomens of dead honeybees showed the Mn signal (El-Jaick et al., 2001). Therefore, the Mn signal do not emit from IGs, possibly from biological tissues (El-Jaick et al., 2001; Esquivel et al., 1999; Krebs and Benson, 1965). Our result of ESCA confirms the presence of SM and FeOOH in IGs. The presence of FeOOH corresponds to that of previous study (El-Jaick et al., 2001).

EPS has been used to study the magnetic material in the whole body of migratory ants (Esquivel et al., 1999; Krebs and Benson, 1965; Wajnberg et al., 2000), honeybees (El-Jaick, et al., 2001), and stingless bees (Lucano et al., 2006). All of the typical X-band resonances spectra show the presence of high field and low field. The signal of $g = 4.3$ at low field shows the presence of the Fe^{+3} ion. The signal of $g = 2.0$ at high field shows the presence of magnetic nanoparticles. The typical X-band resonance spectra in this study are similar to that of previous study (El-Jaick, et al., 2001) and show that honeybees contain SM. However, one derives from naturally dead honeybees; the other derives from a huge amount of purified IGs in trophocytes. So far, IGs are found only in the trophocytes. Therefore, both of X-band resonance spectra should be derived from the SM in IGs.

The temperature dependencies of the HF linewidth of purified IGs spectra are given in Figure 6A. Based on the assumption of isolated spherical nanoparticles, the HF linewidths temperature dependencies are fitted using the equation

$$\Delta H_{pp} = \Delta H^0 \tanh(\Delta E / 2k_B T) \quad (1)$$

where ΔH^0 is the low temperature limit value, ΔE is the magnetic energy KV , V is the nanoparticle magnetic volume, and k_B is the Boltzman constant. The solid lines in Figure 6A are the best fitting curves for $\Delta H^0 = 1791 \pm 37$ Oe and $\Delta E / 2k_B = 50.8 \pm 2.6$ K, respectively. In equation 1, $\Delta H^0 = 5g\beta S n / D3$, the description of the prefactor ΔH^0 includes the g factor (g), the Bohr magneton (β), the spin associated with each magnetic center inside the nanoparticle (S),

the number of magnetic centers per particle (n), and the particle-particle distance in the matrix (D). Assuming $g = 2$, $S = 2$ (Wajnberg, et al., 2000), and $n = 8.5 \times 10^3$ (Hsu and Li, 1994), we obtain $D = 10 \pm 0.1$ nm. An average shift of 2496 ± 50 Oe between the two H_R versus T curves of purified IGs was obtained (Figure 6B). As K is temperature-dependent, ΔE is taken as $K_{\text{ef}}V$ with $K_{\text{ef}} = Ms(-HA)/2$, where (HA) is the mean value obtained from Figure 6C (125 Oe) according to the following equation and previous studies (El-Jaick, et al., 2001; Wajnberg, et al., 2000; Abraçado et al., 2005).

$$H_R = \varpi_R/\gamma - H_D - H_A \quad (2)$$

Taking the magnetite saturation magnetization (Ms) as 470 Oe, magnetic volumes are estimated to be $(0.48 \pm 0.02) \times 10^3 \text{ nm}^3$ and correspond to mean particle diameters of 9.7 ± 0.2 nm. The size of SM in purified IGs is consistent with SM reported by previous studies of the abdomen with SQUID (Lucano et al., 2006), EPR (Ferreira et al., 2005) and HRTEM (Hsu and Li, 1994).

In this study, we demonstrate that SM is present in IGs, which should play a role as magnetoreceptor. More works need to be done to further elucidate the relationship between magnetoreceptor (SM) and magnetoreception in honeybees.

Materials and methods

Honeybees, *Apis mellifera*, were bred in an open environment of a bee-breeding room at the 10th floor of our institute building. Sucrose and pollen grains were occasionally added to

the hives as dietary supplements.

Purification of IGs

Two thousand worker bees were freshly collected from a hive, anesthetized with ice-distilled water, washed with ice-distilled water three times, and dissected with stainless tools. Ventral abdomens were obtained, pollen washed out through stainless filter with honeybee saline (156.4 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 22.2 mM glucose, pH 7.3) three times, and centrifuged against a stainless network filter in a centrifugal tube at 3000 x g for 2 minutes three times. The abdomens were pressed by stainless bar, dissected into small pieces, and centrifuged against a stainless network filter in a centrifugal tube at 15000 x g for 5 minutes three times in honeybee saline containing 0.5 M sucrose and 0.5 M NaCl. Trophocytes were collected into 10 ml honeybee saline containing 0.5 M sucrose, 0.5 M NaCl and 50 µl phenylmethylsulfonyl fluoride, and homogenized by polytron at max speed 30,000 rpm for 3 minutes. The homogenized solution was filtered through a series of filters containing 350 meshes stainless network filter, 10 µm plastic network filter, 20µm membrane filter, plastic network filters with 5 µm and 1 µm, as well as membrane filters with 10 µm, 8 µm, 5 µm, 3 µm, 1.2 µm, 0.8 µm, and 0.65 µm. The filtered solution was centrifuged against 6.0 m sucrose solutions at 23000 x g for 1 hour. The precipitate was collected, resuspended in honeybee saline, and centrifuged against 6.0 m sucrose solutions at 23000 x g for 1 hour again. The precipitate was collected, sucrose washed out with honeybee saline, and

centrifuged against a 0.22 μm membrane filters in a centrifugal tube at 23000 x g for 10 minutes. The precipitate on 0.22 μm membrane filters was collected and analyzed (Hsu, 2006).

Transmission electron microscopy

The purified precipitates were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer with 0.35 M sucrose at pH 7.4 for 30 minutes at 25°C and were postfixed in 1% osmium tetroxide in a 0.1 M phosphate buffer with 0.35 M sucrose at pH 7.4 for 1 hour at 25°C. The purified precipitates were dehydrated through an ethanol series and embedded in Spurr's resin. Thin sections (60-90 nm in thickness) were cut with a diamond knife, stained with uranyl acetate and lead citrate and then examined, using a JEOL JEM-2000EXII transmission electron microscope (TEM), operating at an accelerated voltage of 100 kV. The elemental composition and crystal structure in selected areas of thin sections were analyzed by energy-dispersive X-ray (EDX) microanalysis and by selected-area electron diffraction in a JEOL JEM-2000FX scanning transmission electron microscope (STEM) operating at an accelerated voltage of 100 kV (Hsu and Li, 1993).

SQUID magnetometer

The purified IGs from 2 thousand bees were lyophilized with a lyophilizer for six runs. The resulting powder was transferred to tubes and sealed under nitrogen flux to prevent oxygen contributions to the SQUID signal at low temperatures. The magnetization and

hysteresis of each sample was measured at a temperature of 300 K and magnetic field intensity from -5000 to 5000 Gause under a SQUID magnetometer (Quantum Design MPMS2).

AFM/MFM

The purified IGs from 8 thousand bees were prepared according to TEM techniques. Sections (100 nm in thickness) were cut with a diamond knife, and then were transfer to silicon chips. A commercially available AFM/MFM (SPA300, Seiko Scientific Instrument, Tokyo, Japan) was used to generate the images. The microscope was operating in Tapping/Lift mode to obtain images of both topography and magnetic force over a region of the samples. High-resolution AFM images of the sample surface topography and magnetic interaction was provided simultaneously.

ESCA

The purified IGs from 40 thousand bees were lyophilized with a lyophilizer. The resulting powder was pressed into a disk and characterized by ESCA on a Perkin Elmer Phi 1600 ESCA system. Each sample was analyzed by survey and high-resolution spectra employing an Al K α X-ray anode, energy 1486.6 eV. From the survey spectra, the qualitative and quantative elemental composition and state of the uppermost layer were computed. The binding state of elements Fe was obtained by acquisition of high-resolution spectra and subsequent curve-fitting.

EPR

The purified IGs from 80 thousand bees were lyophilized with a lyophilizer. The resulting powder was transferred to EPR quartz tubes and sealed under nitrogen flux to prevent oxygen contributions to the EPR signal at low temperatures. Measurements were performed in a commercial X-band EPR spectrometer (Bruker EMX-10) operation at a microwave power of 20 mW, with a 100 kHz modulation field of about 1.6 Oe (2 A m^{-1}) in amplitude from 77 to 250 K and 4 to 300 K, respectively. Spectra double integration was obtained using WINEPR software (Bruker). The purified IGs spectra were fitted using Origin 7.5 with the HF. Gaussian and Lorentzian derivative-shaped lines were used to obtain the temperature dependence of the resonance linewidth (ΔH_{pp}), and resonance field (H_R).

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Figure legends

Fig. 1. IGs purified from the trophocytes of honeybees.

A. Numerous IGs appear in precipitates purified from the trophocytes of honeybees. Scale bar, 1 μm .

B. Purified IGs enclosed with lipid-bilayer membranes (arrowhead), similar to that of iron deposition vesicles in the trophocytes. Scale bar, 100 nm.

Fig. 2. The magnetic properties determined by SQUID. An averaged magnetization curve of SQUID from the purified IGs of 2000 honeybees at 300° K for six runs.

Fig 3. The magnetic properties determined by AFM/ MFM.

A. An image of AFM from one of purified IGs of 8,000 honeybees.

B. An image of MFM from one of purified IGs of 8,000 honeybees.

Fig 4. The magnetic properties determined by ESCA.

A. A spectrum of ESCA from the purified IGs of 40,000 honeybees. The left thin line shows the $\text{Fe}(2p_{1/2})$ of FeOOH . The middle thin line shows the $\text{Fe}(2p_{3/2})$ of Fe_2O_3 . The right thin line shows the $\text{Fe}(2p_{1/2})$ of FeO .

B. A photoelectron spectrum of elemental composition of ESCA from the purified IGs of 40,000 honeybees.

Fig 5. The magnetic properties determined by EPR. Typical X-band resonance spectra from the purified IGs of 80,000 honeybees at different temperatures.

Fig 6. The resonance linewidth and anisotropy fields of the broad resonance lines (HF) of purified IGs.

A. Temperature dependence of the resonance linewidth of the HF. The solid line is the best fit of the HF data according to Eq. 1, with $\Delta H^0 = 1791 \pm 37$ Oe and $\Delta E/2k_B = 50.8 \pm 2.6$ K.

B. Temperature dependence of the resonance field of HF and LF. Note that the HF and LF curves are almost parallel to each other, with the field shifting by an average value of about 2496 ± 50 Oe.

C. Anisotropy fields calculated from the resonant field's values, using Eq. 2 for the HF lines.

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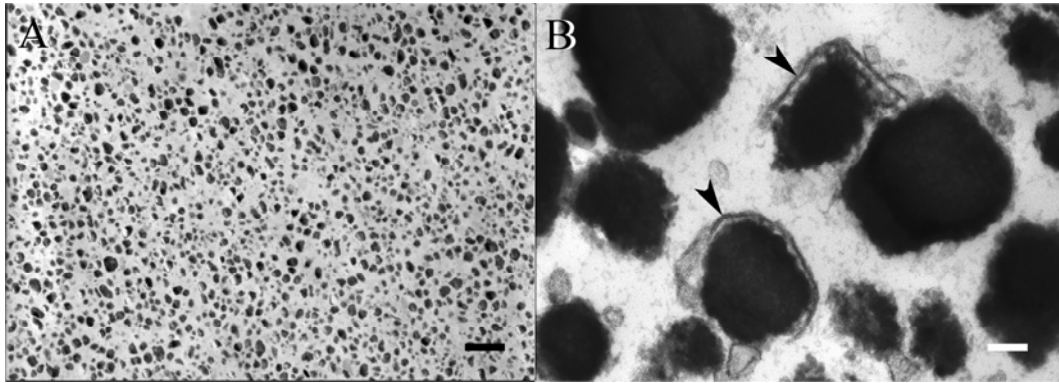


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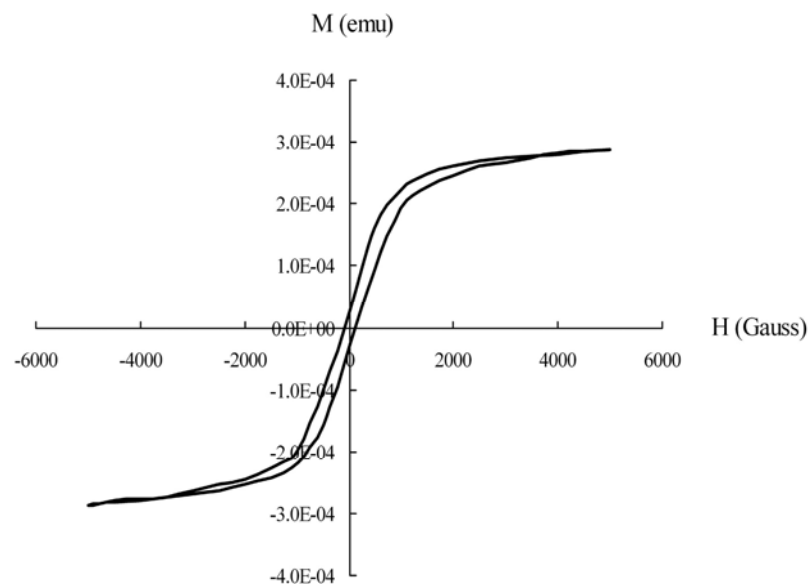


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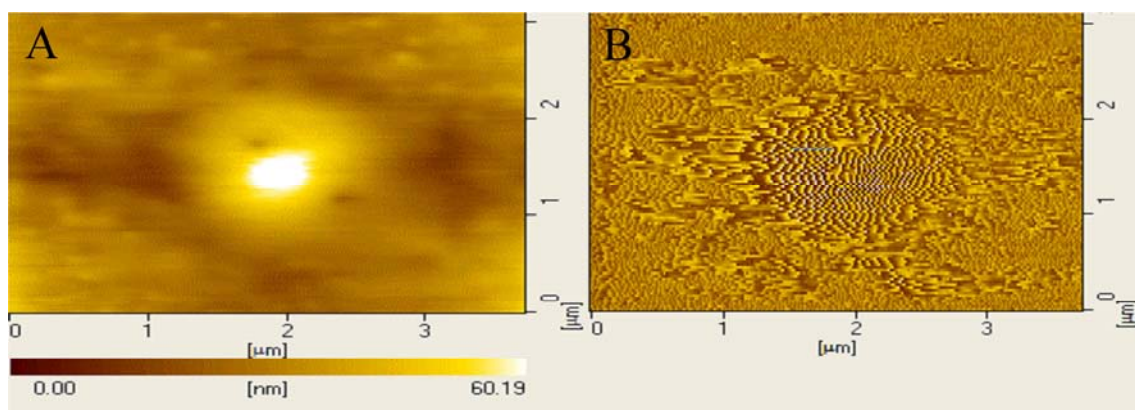


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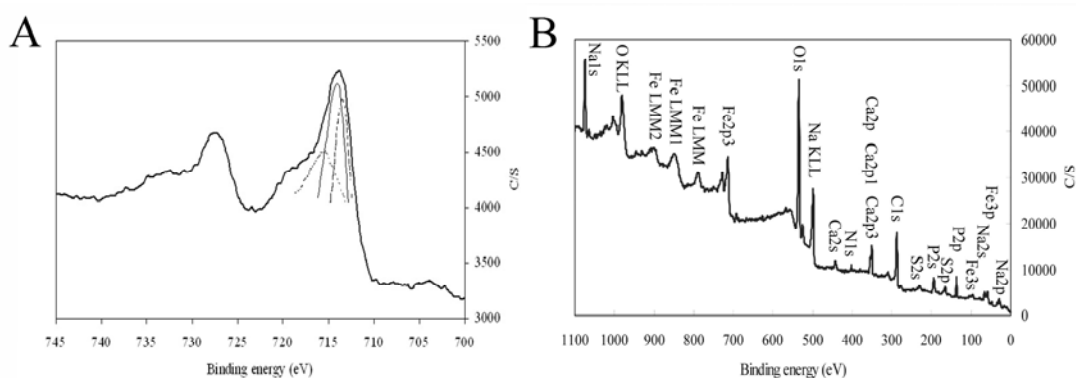


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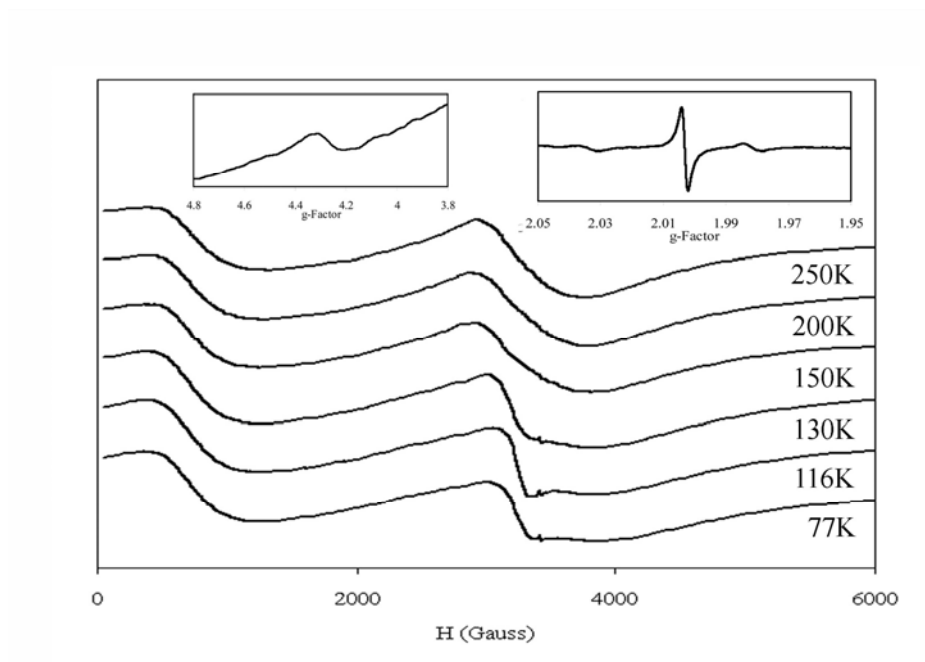


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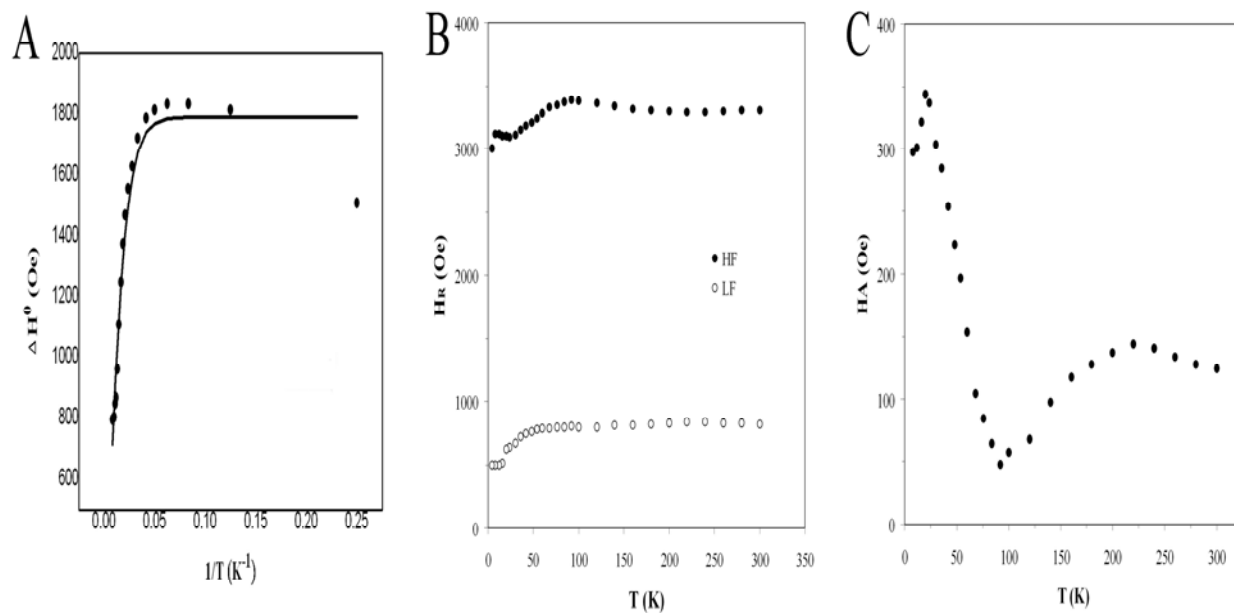


Fig 6. The resonance linewidth and anisotropy fields of the broad resonance lines (HF) of purified IGs.

A. Temperature dependence of the resonance linewidth of the HF. The solid line is the best fit of the HF data according to Eq. 1, with $\Delta H^0 = 1791 \pm 37$ Oe and $\Delta E/2k_B = 50.8 \pm 2.6$ K.

B. Temperature dependence of the resonance field of HF and LF. Note that the HF and LF curves are almost parallel to each other, with the field shifting by an average value of about 2496 ± 50 Oe.

C. Anisotropy fields calculated from the resonant field's values, using Eq. 2 for the HF lines.