

Harnessing the Extracellular Bacterial Production of Nanoscale Cobalt Ferrite with Exploitable Magnetic Properties

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ABSTRACT Nanoscale ferrimagnetic particles have a diverse range of uses from directed cancer therapy and drug delivery systems to magnetic recording media and transducers. Such applications require the production of monodisperse nanoparticles with well-controlled size, composition, and magnetic properties. To fabricate these materials purely using synthetic methods is costly in both environmental and economical terms. However, metal-reducing microorganisms offer an untapped resource to produce these materials. Here, the Fe(III)-reducing bacterium *Geobacter sulfurreducens* is used to synthesize magnetic iron oxide nanoparticles. A combination of electron microscopy, soft X-ray spectroscopy, and magnetometry techniques was employed to show that this method of biosynthesis results in high yields of crystalline nanoparticles with a narrow size distribution and magnetic properties equal to the best chemically synthesized materials. In particular, it is demonstrated here that cobalt ferrite (CoFe₂O₄) nanoparticles with low temperature coercivity approaching 8 kOe and an effective anisotropy constant of $\sim 10^6$ erg cm⁻³ can be manufactured through this biotechnological route. The dramatic enhancement in the magnetic properties of the nanoparticles by the introduction of high quantities of Co into the spinel structure represents a significant advance over previous biomineralization studies in this area using magnetotactic bacteria. The successful production of nanoparticulate ferrites achieved in this study at high yields could open up the way for the scaled-up industrial manufacture of nanoparticles using environmentally benign methodologies.

KEYWORDS: cobalt ferrite · *Geobacter sulfurreducens* · Fe(III)-reducing bacteria · nanoparticles · magnetism

The production of ferromagnetic nanoparticles for pioneering cancer therapy, drug delivery, chemical sensors, catalytic activity, photoconductive materials, as well as more traditional uses in data storage embodies a large area of inorganic synthesis research.^{1–4} In particular, the addition of transition metals other than Fe into the structure of magnetite (Fe₃O₄) has been shown to greatly enhance the magnetic properties of the particles, tailoring them to different commercial uses.^{1,2,5} However, synthesis of magnetic nanoparticles is often carried out at high temperatures with toxic solvents resulting in high environmental and energy costs. Additionally, these ferrite nanoparticles are not in-

trinsically biocompatible, and to make them suitable for insertion into the human body is a rather intricate task.

A relatively unexplored resource for magnetic nanomaterial production is subsurface Fe(III)-reducing bacteria, as these microorganisms are capable of producing large quantities of nanoscale magnetite (Fe₃O₄) at ambient temperatures. Metal-reducing bacteria live in environments deficient in oxygen and conserve energy for growth through the oxidation of hydrogen or organic electron donors, coupled to the reduction of oxidized metals such as Fe(III)-bearing minerals.⁶ This can result in the formation of magnetite *via* the extracellular reduction of amorphous Fe(III)-oxyhydroxides causing the release of soluble Fe(II) and resulting in complete recrystallization of the amorphous mineral into a new phase.^{7,8} Some previous studies have reported altering the composition of biogenic magnetite produced by Fe(III)-reducing bacteria for industrial and environmental applications.^{9,10} However, research into the commercial exploitation of bacteria to form magnetic minerals has focused primarily on magnetotactic bacteria which form magnetosomal magnetite internally using very different pathways to those bacteria forming magnetite outside the cell.^{11–14} Magnetotactic bacteria live at the sediment–water interface and use internal nanomagnets to guide them to their preferred environmental niche using the Earth's magnetic field. Since magnetotactic bacteria generally grow optimally under carefully controlled microaerobic conditions, the culturing processes for these organisms are challenging and result in low yields of nanomagnetite. Despite these limitations, magnetotactic bacteria

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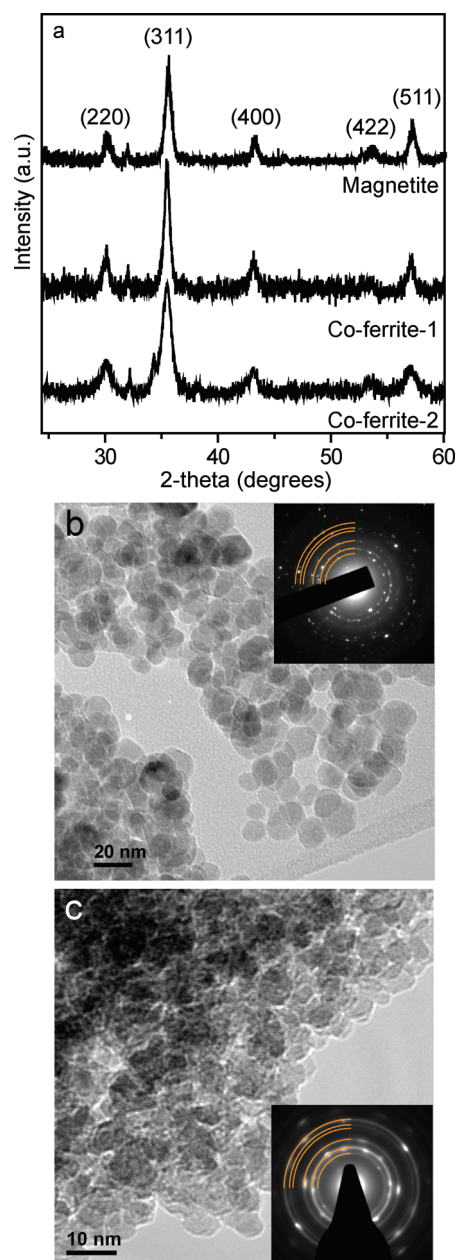


Figure 1. (a) X-ray diffraction of biogenic magnetite, Co-ferrite-1, and Co-ferrite-2. Transmission electron microscopy (TEM) with selected area diffraction (inset) for biogenic (b) magnetite and (c) Co-ferrite-2. Note the different scale bars of the TEM images.

have been shown to incorporate $\sim 1\%$ Co into the magnetite structure *in vivo*,¹⁵ and CoFe_2O_4 was synthesized

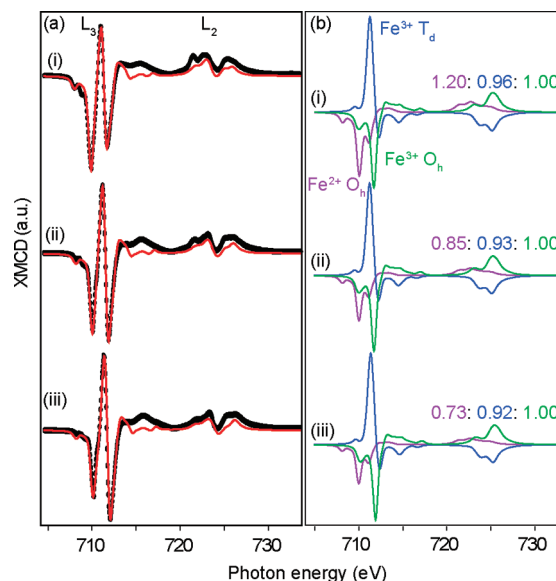


Figure 2. (a) Experimental Fe $L_{2,3}$ XMCD spectra (black circles) with best-fit spectra (red line) for biogenic (i) magnetite, (ii) Co-ferrite-1, and (iii) Co-ferrite-2. (b) For the best-fit spectrum of each biogenic sample, the decomposition into the spectra for the $\text{Fe}^{2+} O_h$, $\text{Fe}^{3+} T_d$, and $\text{Fe}^{3+} O_h$ components is given. The color-coded numbers indicate the proportions of the components, and the error bar for each site occupancy is ± 0.02 cations.

in vitro,¹⁴ altering the magnetic properties of the material formed. Although these previous studies are an important first step, in order to obtain the degree of control over the magnetic properties required by potential applications, Co must be incorporated into the spinel structure together with high nanoparticle yields. It is not clear at present how this could be achieved using the highly regulated intracellular magnetosome systems.

Here we present an alternative and efficient method to produce large quantities of highly crystalline magnetite and cobalt ferrite nanoparticles using the Fe(III)-reducing bacterium, *Geobacter sulfurreducens*, at ambient temperatures through the extracellular dissimilatory reduction of Fe(III)-oxyhydroxides without and with addition of cobalt. A suite of three samples containing progressive additions of Co was produced for this study. A combination of transmission electron microscopy (TEM), X-ray diffraction (XRD), X-ray absorption (XA), and X-ray magnetic circular dichroism (XMCD) was used to determine the crystallographic structure,

TABLE 1. Structural and Magnetic Properties from Biogenic Magnetite and Co-Enriched Bioferrites

sample	biomagnetite	Co-ferrite-1	Co-ferrite-2
EDX Co/Fe ratio	0.00:1	0.06:1	0.30:1
XRD crystallite size (nm)	15	16	8
saturation magnetization, M_s (emu g^{-1}) at 298 K	78	—	48
remanent magnetization, M_r/M_s at 298 K/at 5 K	0.06/0.25	—	0.04/0.62
coercivity, H_c (Oe) at 5 K	360	—	7900
median particle diameter, d_0 (nm)	—	—	7.9
most probable diameter, d_p (nm)	—	—	6.8
dispersion index, σ_d	—	—	0.38

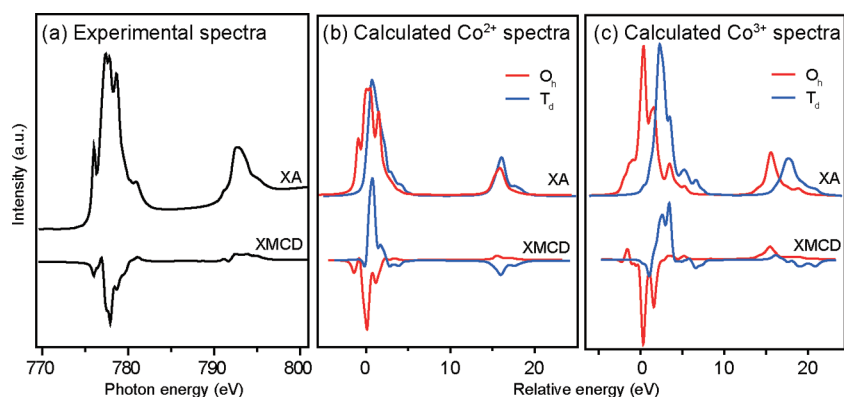


Figure 3. (a) Experimental $\text{Co } L_{2,3}$ XA and XMCD spectra for biogenic Co-ferrite-2. Calculated XA and XMCD spectra for (b) Co^{2+} and (c) Co^{3+} in octahedral (red line) tetrahedral (blue line) coordination. Calculations were done following the method of refs 18 and 19.

site occupancies, and valence states of the Fe and Co cations within the spinel structure. Furthermore, superconducting quantum interference device (SQUID) magnetometry was used to determine the magnetic properties of these materials. The exceptional ability of the bacteria to manufacture a wide range of useful nanomaterials has until now been overlooked for production on a commercial scale.

RESULTS AND DISCUSSION

Biogenic magnetite and two related materials (biogenic Co-ferrite-1 and biogenic Co-ferrite-2) were produced from starting materials of Fe(III)-oxyhydroxide containing increasing amounts of co-precipitated Co(II), at ambient temperature and pressure using the metal-reducing bacterium *Geobacter sulfurreducens*. Conversion of the amorphous Fe(III)-oxyhydroxide occurred during the reductive transformation by *G. sulfurreducens* to give a biomineral phase enriched with Fe(II), while control experiments without cells showed no change in acid-extractable Fe(II) (see Supporting Information). The yield of nanoparticles in this experiment was 1.6 g per 100 mL per day, which is comparable to or greater than yields given by other bacterial synthesis methods.^{14,16} Energy dispersive X-ray (EDX) analysis of the particles indicated that the Co content of the total cation distribution was 6 atom % for Co-ferrite-1 and 23 atom % for Co-ferrite-2. The latter is close to the Co content of stoichiometric Co-ferrite (CoFe_2O_4). X-ray diffraction (XRD) and selected area electron diffraction (SAED) in Figure 1 show that each of the samples gives only reflections arising from a ferrite structure. Interestingly, it was found that the particle size decreases for increasing Co content, as revealed by an overall broadening of the individual diffraction peaks in Figure 1a. Fitting the (311) diffraction peak with a Lorentzian line shape using the Scherrer equation¹⁷ gives the crystallite size for each material (Table 1). The XRD shows that the material is nanocrystalline, where the addition of 23 atom % Co into the structure reduces the average crystallite size from 15–16 to 8 nm. These

values correlate well with the observed particle sizes in TEM images (Figure 1), confirming the single crystalline nature. In addition, the TEM images—as well as magnetization measurements discussed below—indicate a narrow size distribution of the particles, crucially important for many technological applications.

The cation distribution in the ferrite nanoparticles was investigated using X-ray magnetic circular dichroism (XMCD) at the Fe $L_{2,3}$ and Co $L_{2,3}$ X-ray absorption edges. XMCD is the difference in the absorption of circularly polarized X-rays for opposite sample magnetization directions. Stoichiometric magnetite is an inverse spinel, which contains tetrahedral (T_d) and octahedral [O_h] sites accommodating Fe^{2+} and Fe^{3+} cations according to the formula $(\text{Fe}^{3+})_{T_d}[\text{Fe}^{2+}\text{Fe}^{3+}]_{O_h}\text{O}_4$. Each specific cation in the spinel structure generates a unique XMCD signature that is determined by its valence state (number of d electrons), site symmetry (*i.e.*, T_d or O_h), and magnetization (local moment) direction. The XA and XMCD spectra of the individual cations can be computed using atomic multiplet calculations.^{18,19}

By fitting a weighted sum of these calculated spectra to the measured XMCD, the site distribution of the Fe cations can be obtained (for details see ref 20). Figure 2a shows the data together with the calculated fits for the Fe $L_{2,3}$ spectra of the biomagnetite and Co-enriched bioferrite samples. Figure 2b gives the relative proportions of the three Fe components, $\text{Fe}^{2+} O_h$, $\text{Fe}^{3+} T_d$, and $\text{Fe}^{3+} O_h$, which primarily make up the intensities of the three main peaks (negative, positive, and negative, respectively) in the Fe L_3 edge (~ 708 – 714 eV). A comparison of the biogenic materials shows that the most striking change with increasing Co amount is the gradual decrease in intensity of the leading negative peak in the Fe L_3 edge, corresponding to a decrease in $\text{Fe}^{2+} O_h$ content. The best-fit spectra give the values for the relative site occupancies as indicated in Figure 2b. The $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio in biogenic magnetite is 1.22:2, which is higher than the 1:2 ratio expected for stoichiometric magnetite, which is due to an increased amount of $\text{Fe } d^6 O_h$ as a result of the anoxic environment in which the magnetite was formed.^{20,21} Upon the addition of Co, the amount of Fe^{2+} decreases rapidly, with only a small accompanying change in the amount of Fe^{3+} cations per formula unit. This implies that Co is predominantly replacing Fe^{2+} cations in octahedral sites.

The site occupancy and oxidation state of the Co can be directly assessed by examining the Co $L_{2,3}$ XA and XMCD spectra (Figure 3). The close similarity between the spectra for Co-ferrite-2 and synthetically produced CoFe_2O_4 thin films²² signifies that the bacteria were able to suitably accommodate Co in the ferrite

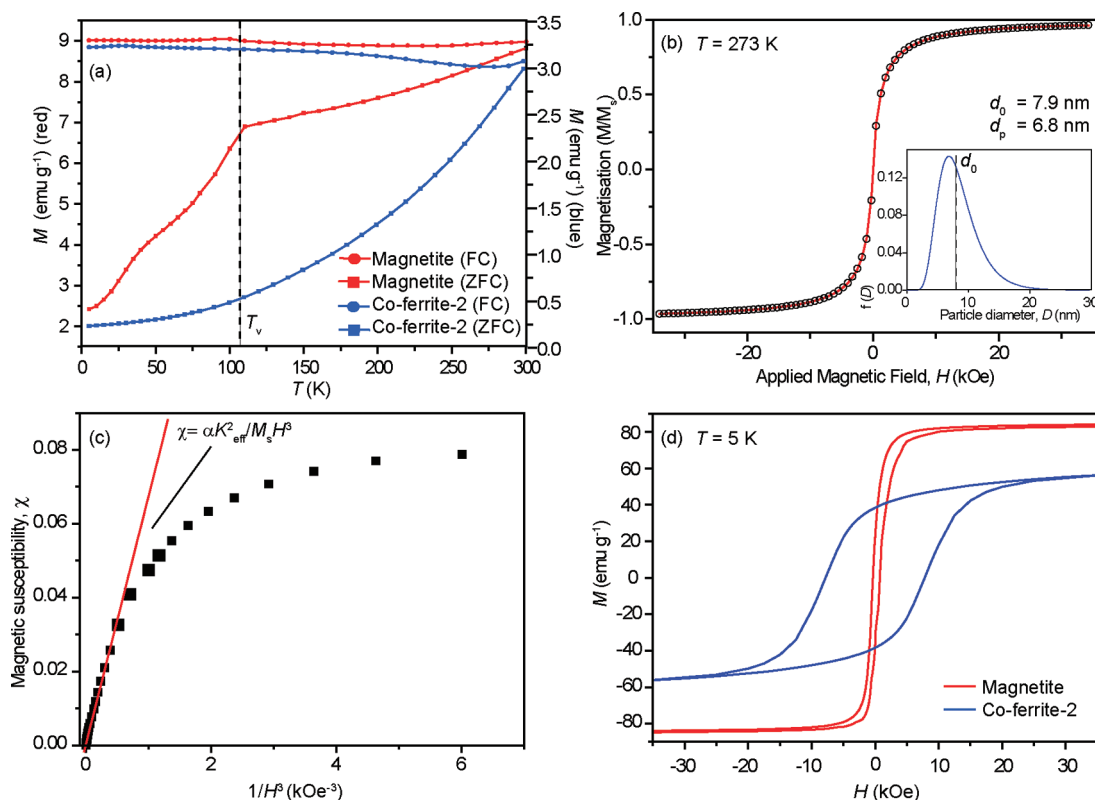


Figure 4. SQUID magnetometry measurements. (a) Zero-field cooled (ZFC) and field cooled (FC) curves for biogenic magnetite (red lines) and biogenic Co-ferrite-2 (blue lines). (b) Magnetic hysteresis curve at 273 K for Co-ferrite-2 with Langevin function fitting and (inset) derived particle size distribution. (c) Fit for biogenic magnetite using the Law of Approach to Saturation. (d) Magnetic hysteresis curves taken at 5 K for biogenic magnetite (red line) and Co-ferrite-2 (blue line).

structure. Calculated XA and XMCD spectra for Co²⁺ (Figure 3b) and Co³⁺ (Figure 3c) in both O_h and T_d coordination have been normalized and broadened to account for instrumental broadening for comparison with experimental spectra (Figure 3a). The strong resemblance of the experimental data to the calculated Co²⁺ O_h XA and XMCD suggests that the Co cations reside primarily on these sites, particularly as the magnetic moments on the T_d and O_h sites are antiferromagnetically coupled, resulting in an XMCD with opposite sign for the two sites. The measurements show that the sign of the Co XMCD is the same as that of the Fe O_h XMCD, hence confirming the O_h site occupancy of Co. Further confirmation arises from the fact that the presence of the Co²⁺ O_h coincides directly with the decrease in Fe²⁺ on octahedral sites found from the Fe $L_{2,3}$ XMCD. Nevertheless, a small amount ($\sim 10\%$) of Co residing in a different site or as Co³⁺ cannot be discounted as the fit is not perfect, particularly at the high energy side of the L_3 .

The magnetic properties of the nanoparticles, graphically represented in Figure 4, are governed by the anisotropy energy, which constrains the nanoparticle magnetization to align along a specific direction known as the easy axis. The maximum anisotropy energy for a particular nanoparticle depends on the product of its volume, V , and anisotropy constant, K . Above the so-called blocking temperature, T_B , the thermal energy is sufficient to surmount the anisotropy energy

barrier and the nanoparticle magnetization is free to align in arbitrary directions. Thus, for an assembly of such nanoparticles, the net magnetization measured in the absence of an applied magnetic field (*i.e.*, the remanent magnetization of the sample) will average out to zero and the particles are said to be in superparamagnetic state.

An estimate for the blocking temperature can typically be obtained from peaks in the field cooled (FC) and zero-field cooled (ZFC) magnetization curves.²³ The experimental results for the biogenic magnetite and Co-ferrite-2 samples, obtained by SQUID magnetometry, are shown in Figure 4a. In this case, no peaks can be discerned in the ZFC/FC curves below 300 K, suggesting that the blocking temperature is close to room temperature for both samples. The remanent magnetization for the Co-ferrite-2 is lower than for biomagnetite (Table 1), and indeed, this sample displays the typical superparamagnetic magnetization curve at room temperature, as shown in Figure 4b.

The biogenic magnetite retains a small residual coercivity at room temperature (not shown), indicating an incomplete superparamagnetic state. The FC and ZFC curves in Figure 4a for this sample contain a sharp feature at ~ 110 K that is due to the Verwey transition (T_v),²⁴ which is a well-known but poorly understood characteristic of pure Fe₃O₄ observed between 100 and 120 K depending on the exact composition of the ma-

terial.²⁵ This phase transition remains frequently unobserved in synthetic nanoparticulate magnetite,²⁵ possibly due to lack of crystalline coherence compared to the bulk. The presence of a sharp transition temperature for the biogenic magnetite is a clear indication of the high quality crystal growth obtained by the biomineralization route, even for nanoparticles below 20 nm. The absence of the transition for the Co-ferrite-2 sample, on the other hand, corroborates the finding that the Co entered the spinel structure.²⁶

The exact shape of the magnetization curve for superparamagnetic particles depends on the magnetic particle volume and can be described by a Langevin function.^{25,27} For an assembly of polydisperse particles, the magnetic particle size distribution can be obtained from a fit to the magnetization curve taking a weighted sum over Langevin functions.^{28,29} Using the best-fit curve shown in Figure 4b for the Co-ferrite-2 sample, the log-normal distribution is obtained (inset). It is seen that the median (7.9 nm) and most probable particle diameter (6.8 nm) of the log-normal distribution agrees well with the respective average crystallite size measured by XRD (Table 1) and particle size imaged in TEM (Figure 1). This again supports the notion that the particles are highly crystalline with little or no surface disorder. Furthermore, the dispersion index determined (Table 1) is comparable with chemically produced Co-ferrite particles of similar size.³⁰

An estimation of the effective anisotropy constant, K_{eff} , for the samples can be obtained by using the Law of Approach to Saturation.^{31,32} This allows us to deduce K_{eff} from a fit to the magnetic susceptibility, χ , versus H^{-3} curve, where H is the applied magnetic field, as is shown for the biomagnetite sample in Figure 4c. Measurements were carried out at low temperature (5 K, *i.e.*, well below the blocking temperature) to ensure the samples are in the ferrimagnetic state. Assuming uniaxial anisotropy, the fits give $K_{\text{eff}} = 2.4 \times 10^5 \text{ erg cm}^{-3}$ for biomagnetite and $K_{\text{eff}} = 2.3 \times 10^6 \text{ erg cm}^{-3}$ for Co-ferrite-2. Thus, the Co inclusion in the spinel increases the magnetic anisotropy by an order of magnitude, which is consistent with the presence of Co^{2+} in octahedral sites found from the XMCD measurements and has also been observed for synthetic Co-ferrite particles.^{5,33} The results obtained for K_{eff} are also comparable to the values of the first-order anisotropy constant for bulk magnetite ($|K_1| \cong 2.7 \times 10^5 \text{ erg cm}^{-3}$ for

the low temperature phase)³¹ and bulk Co-ferrite, CoFe_2O_4 ($|K_1| \sim 2 \times 10^6 \text{ erg cm}^{-3}$).³⁴

The coercivity, H_C , of the samples (*i.e.*, the magnetic field needed for the magnetization to return to zero) can be related to the anisotropy constant according to $H_C = 0.958 K/M_s^{35}$ (assuming uniaxial anisotropy), where M_s is the saturation magnetization (Table 1). At 5 K, this gives a coercivity of ~ 500 and ~ 7000 Oe for biomagnetite and Co-ferrite-2, respectively. The measured values (shown in Figure 4d and Table 1) are comparable to these estimated values derived from the measured anisotropy constants. However, it should be noted that the coercivity of 7000 Oe obtained above for the Co-ferrite-2 sample underestimates the experimental value of 7900 Oe. This is because, even at the highest measurement field of 7 T, the sample was not completely saturated and therefore the Law of Approach to Saturation can provide only a lower limit to the effective anisotropy constant in this case. Further, we cannot rule out a contribution to the approach to magnetic saturation from noncollinear surface spins,³⁶ which would tend to make the surface of the particles magnetically hard compared to the inner core.

From the FC/ZFC curves shown in Figure 4a, it can be seen that the Co-ferrite-2 sample is only just superparamagnetic at room temperature (*i.e.*, the blocking temperature is close to room temperature). A modest increase in either the particle diameter or the anisotropy constant (*e.g.*, by slightly increasing the Co content) should be sufficient to produce nanoparticles with ferrimagnetic properties and large coercivities suitable for applications above room temperature. This is the subject of further work and will be presented elsewhere.

In summary, we have shown a dramatic enhancement in the magnetic properties of biogenically produced nanoparticles by introducing large quantities of Co into the spinel structure, which represents a major advance over previous biomineralization studies in this area. Inclusion of other transition metals into the spinel structure by Fe(III)-reducing bacteria to tailor the magnetic properties of nanoferrites could lead to a suite of materials required for different technological uses. The successful production of highly ordered crystalline nanoparticulate ferrites achieved here shows the potential for scaled-up industrial manufacture of nanoparticles using environmentally benign methodologies.

METHODS

Synthetic amorphous Fe(III)-oxyhydroxide was produced using the method of Lovley and Phillips,³⁷ where a 0.4 mol solution of FeCl_3 is neutralized by 10 N NaOH to pH 7 and the solid then washed in deionized H_2O until no Cl^- ions remain. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ was added prior to neutralization at a concentration of either 0.02 or 0.132 mol to create FeCo-oxyhydroxides which formed a suite of starting materials with an increasing amount of cobalt which result in samples Co-ferrite-1 and Co-

ferrite-2, respectively. The starting material was characterized using XRD to confirm that poorly crystalline material had formed. *Geobacter sulfurreducens* was obtained from our laboratory culture collection at the Williamson Centre for Molecular Environmental Science at the University of Manchester and grown under strictly anaerobic conditions at 30 °C in modified fresh water medium.³⁸ Sodium acetate (20 mmol) and fumarate (40 mmol) were provided as the electron donor and acceptor, respectively. All manipulations were done under an atmosphere of $\text{N}_2 - \text{CO}_2$

(80:20). Late log-phase cultures of *G. sulfurreducens* were harvested by centrifugation at 4920g for 20 min and washed twice in bicarbonate buffer (NaHCO₃ 30 mmol, pH 7.1) under N₂-CO₂ (80:20) gas prior to use. Aliquots of the washed cell suspension (1.5 mL) were added to sealed anaerobic bottles containing 28.5 mL of bicarbonate buffer to a final concentration of bacteria of ~0.2 mg protein per mL. The following additions were made from anaerobic stocks as required: an Fe(III)-oxyhydroxide or CoFe(III)-oxyhydroxide suspension (10 mmol L⁻¹), sodium acetate (10 mmol), and anthraquinone 2,6-disulfonate (10 μmol). Bottles were incubated in the dark at 20 °C; 100 μL of slurry was removed from each serum bottle periodically, using aseptic and anaerobic technique and analyzed for 0.5 mol HCl-extractable Fe²⁺ employing the ferrozine method.³⁷ After ferrite formation, samples were washed twice in deionized water to remove cells and buffer solution. Aliquots of the sample suspension were then dropped on carbon-coated copper grids and left to dry prior to introduction into the microscope.

Transmission electron microscopy (TEM) was carried out using a Phillips/FEI Tecnai electron microscope equipped with a field emission gun (FEG), EDX system (Oxford Instruments UTW ISIS), and Gatan imaging filter (GIF). All TEM images presented here are bright-field images obtained using an operating beam voltage of 300 keV. Selected area electron diffraction (SAED) patterns were acquired using an appropriate diffraction aperture. All (powder) X-ray diffraction (XRD) measurements were acquired over a 2θ range of 10–70° with a step size of 0.02° using a Bruker D8Advance with Cu Kα₁ source.

X-ray absorption (XA) spectra at the Fe and Co L_{2,3} edges were obtained on beamline 4.0.2 at the Advanced Light Source (ALS), Berkeley, CA, using the eight-pole resistive magnet endstation.³⁹ Samples were prepared by washing aliquots of the suspension in deionized water before drying under anaerobic conditions. The powders were then, in an anaerobic cabinet, mounted on carbon tape attached to the sample manipulator. XA was monitored in total-electron yield (TEY) mode, which has an effective probing depth of ~4.5 nm. At each photon energy of the spectrum, the XA was measured for two opposite magnetization directions by reversing the applied field of 0.6 T. After normalization to the incident beam intensity, the XMCD is obtained as the difference between the two XA spectra.⁴⁰ To obtain the cation distribution over the three Fe sites, the experimental XMCD was fitted by means of a nonlinear least-squares analysis, using the calculated spectra for each site. In these calculations, described in refs 18 and 19, the 10Dq crystal field parameters were taken as 1.2 and -0.6 eV for Fe O_h and T_d sites and 0.9 and -0.5 eV for Co O_h and T_d sites. The results were convoluted by a Lorentzian of Γ = 0.3 (0.5) eV for the L₃ (L₂) edge to account for intrinsic core-hole lifetime broadening and by a Gaussian of σ = 0.2 eV to account for instrumental broadening.

Magnetic measurements were performed on polycrystalline samples restrained in eicosane, using a Quantum Design MPMS-XL SQUID magnetometer equipped with a 7 T magnet. Zero-field cooled (ZFC) and field-cooled (FC) magnetization curves were recorded over 5–300 K temperature range with an applied magnetic field of 100 Oe. The diamagnetism of the sample holder and of eicosane was measured and extracted from the raw magnetic data.

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Supporting Information Available: Graph of Fe(II) concentration changes with time during the formation of biogenic ferrites. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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