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Understanding the Formation Mechanism of Magnetic Mesocrystals with (Cryo-)Electron Microscopy


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Supporting Information

ABSTRACT: Magnetite \((\text{Fe}_3\text{O}_4)\) nanoaggregates with a flower-like morphology are considered promising materials in the field of magnetically induced hyperthermia in cancer therapy due to their good heating efficiency at low applied alternating magnetic fields. Although the structure and the magnetic state of such flower-like aggregates have been investigated previously, the mechanism that leads to the hierarchical morphology is still poorly understood. Here, we study the formation mechanism of \(\text{Fe}_3\text{O}_4\) aggregates synthesized through the partial oxidation of ferrous hydroxide in the presence of poly(acrylic acid) by using cryogenic electron microscopy. The aggregates are formed through a multistep process involving first the conversion of ferrous hydroxide precursors in ∼5 nm primary particles that aggregate into ∼10 nm subunits, often showing a uniform magnetic state. Finally, the conversion of ferrous hydroxide precursors in ∼10 nm primary particles that aggregate into ∼10 nm subunits, often showing a uniform magnetic state, finally arrange into the secondary mesocrystal structure. High-resolution electron tomography is used to show that the \(\text{Fe}_3\text{O}_4\) mesocrystals are composed of ∼10 nm subunits, often showing a uniform crystallographic orientation resulting in single-crystal-like diffraction patterns. Furthermore, electron holography reveals that mesocrystals have a single magnetic domain despite polymeric interfaces between subunits being present throughout the mesocrystal. Our findings could be used to design materials with specific properties by modulating the morphology and/or magnetic state that is suitable for biomedical application.

INTRODUCTION

The use of specially designed nanoparticles (NPs) for cancer diagnosis and treatment has recently attracted more attention.\(^1\)−\(^3\) In particular, magnetite \((\text{Fe}_3\text{O}_4)\) is considered to be one of the most promising materials as it possesses a high saturation magnetization. Moreover, due to its biocompatibility, magnetite is a contrast agent in magnetic resonance imaging (MRI) and can be used in targeted drug delivery systems.\(^4\)\(^5\)

The magnetic properties of \(\text{Fe}_3\text{O}_4\)-NPs have been exploited to locally induce cell necrosis in magnetically mediated hyperthermia for cancer therapy.\(^6\)−\(^8\) When an external alternating magnetic field is applied, the magnetic domains convert magnetic energy into heat, due to loss of hysteresis, Neél relaxation, or induced Eddy currents.\(^3\)\(^9\) The temperature can be locally increased above 42 °C, which is known to interfere with many cell functions (cell growth and differentiation), which, in turn, leads to apoptosis.\(^10\)\(^11\)

Although the \(\text{Fe}_3\text{O}_4\)-NPs has been successfully employed in \textit{in vivo} hyperthermia therapy for small animals,\(^12\)\(^13\) clinical human studies have been very limited.\(^14\)−\(^16\) The problems of accurate thermometry within the tumor mass, precise tumor heating, preventing damage of the surrounding healthy tissues, and the accumulation of intravenously administrated \(\text{Fe}_3\text{O}_4\)-NPs in healthy tissues, such as liver and kidneys, make the treatment still very challenging.\(^17\) Therefore, recent studies have been focused on optimizing the heating efficiency of the \(\text{Fe}_3\text{O}_4\)-NPs.\(^18\)\(^19\) The heating efficiency is measured in terms of the specific absorption rate (SAR), which in turns depends on the magnetic saturation \((M_s)\) and the volume ratio of the \(\text{Fe}_3\text{O}_4\)-NPs.\(^20\)\(^21\) Magnetite clusters embedded in polymers with a flower-like morphology are the most investigated system.
for magnetically mediated hyperthermia, as they have shown a high SAR at low applied alternating magnetic fields.\textsuperscript{18,22,23}

Magnetite nanoclusters can be synthesized at high temperatures (>90°C) by polyol methods.\textsuperscript{18,23−27} Depending on the specific polymer that is used during synthesis, the crystal size can vary over a wide range (35–200 nm). The resulting magnetite crystals consist of smaller subdomains arranged into a flower-like morphology that are often crystallographically aligned. Such a polydomain crystal showing a single crystal-like diffraction pattern is termed a mesocrystal.\textsuperscript{28−30} Although the structure and the magnetic properties have been well investigated in terms of hyperthermia performance, very little is known about the formation mechanism that leads to such hierarchical crystals. It has been proposed that the superstructure obtained via aqueous polyol synthesis is formed starting from the agglomeration of ferricydrate-like ~2 nm

Figure 1. Cryo-TEM micrographs of the solution at different time points during the reaction. (a−e) Objects and assembled structures found in solution (blue border): (a) green rust platelets, (b) primary particles (white arrows) and primary magnetite crystals (yellow arrows), (c) flower-like magnetite aggregates, (d) star-like green rust aggregates, and (e) green rust platelet network. (f−i) Time series showing the morphological progression during magnetite synthesis (red border): (f) solution at 0 min, showing star-like green rust aggregates arranged in strings, (g) solution at 7 min showing magnetite aggregates arranged in strings together with green rust aggregates (the arrow highlights individual dispersed magnetite aggregates), (h) solution at 45 min, showing the dissolution of the green rust to form magnetite (the inset shows an enlargement of a dissolving green rust platelet), and (i) magnetite aggregate strings. The insets in panels c–f and i are the SAED patterns of the areas shown in the corresponding images. The assignment of reflections for the SAED pattern shown in panel c can be found in Supporting Information section 1 and Figure S1.
primary particles formed in the presence of Fe$^{2+}$ with a mechanism similar to what was reported in 2013 for the coprecipitation of Fe$^{2+}$ and Fe$^{3+}$ at room temperature in the absence of additives. However, there are no in situ observations of the formation mechanism that can confirm this hypothesis. In fact, most studies have been focused on the characterization of the final product with ex situ techniques. Furthermore, intermediate stages during the formation of magnetite nanoflowers have been investigated with only conventional (dry) transmission electron microscopy (TEM), which often introduces artifacts caused by the drying process or by the exposure to oxygen. In contrast, cryogenic TEM (cryo-TEM) can image the solution in its native hydrated state, giving the great advantage of avoiding the formation of these artifacts. 

In this study, we present a time-resolved analysis of the different stages during the formation of flower-like magnetite crystals using cryo-TEM. The magnetite crystals were synthesized using the partial oxidation method in the presence of poly(acrylic acid) (pAA). This is a well-known aqueous synthetic method, involving a ferrous hydroxide precursor. pAA was chosen as an additive because of its well-known affinity for the iron oxide surface, biocompatibility, and water solubility. Furthermore, we have performed high-resolution electron tomography (HR-ET) to resolve the hierarchical three-dimensional (3D) structure and off-axis electron holography (EH) to study the magnetism of the crystals. The results show that magnetite mesocrystals are formed through a multistep process, involving Fe(OH)$_2$ as a precursor and different intermediate structures. Unraveling the formation steps of this hierarchical structure is important in the manipulation of the aggregation steps. We believe that a better understanding of the formation mechanism of this magnetic superstructure will aid the future design of materials with tunable properties for biomedical applications, such as therapeutic agents in cancer treatment.

**RESULTS AND DISCUSSION**

Magnetite nanocrystals were synthesized in water through the partial oxidation of ferrous hydroxide in the presence of pAA as reported by Altan et al. In short, an aqueous solution containing KOH (0.14 M), KNO$_3$ (0.2 M), and pAA (450 kDa, $\sim 4 \times 10^{-6}$ M) was degassed with nitrogen gas for approximately 30 min, and then the mixture was added to an FeSO$_4$ solution (0.026 M). The overall synthesis was performed under a nitrogen flow at 90 °C for $\sim$4 h. A detailed synthesis protocol of Fe$_3$O$_4$-pAA can be found in Materials and Methods. Immediately after the addition of the KOH/KNO$_3$/pAA mixture to the FeSO$_4$ solution, the iron precipitates as green rust, consisting of partially oxidized ferrous hydroxide. Subsequently, magnetite is formed by the oxidation of the green rust, via a dissolution–recrystallization process. Thus, green rust can be considered as both a precursor phase and a reserve of iron ions that have an inherent low solubility in water. Beyond the reaction conditions used in this work, it is well-known that additional factors, such as the concentration of the Fe$_3$O$_4$ base, and oxidant, can influence the final Fe$_3$O$_4$ crystal size and morphology, for which we would like to refer to the work of Vereda et al.

We investigated the evolution of morphology and crystallinity during magnetite formation using cryo-TEM in combination with low-dose selected-area electron diffraction (SAED) (Figure 1 and Supporting Information section 1). For this, samples were prepared at different time points during the reaction by plunge freeze vitrification. Time zero is taken as the moment immediately after mixing of the reagents.

The cryo-TEM micrographs taken at different times show the presence of different objects in solution (Figure 1a–e): green rust platelets (Figure 1a), primary particles (white arrows in Figure 1b), primary magnetite crystals (yellow arrows in Figure 1b), and secondary magnetite aggregates with a flower-like morphology (Figure 1c and Figure S1). At alkaline pH, the surface of the green rust is positively charged while the magnetite surface negative. Because pAA is a negatively charged polyelectrolyte under these conditions, it will adsorb on the green rust surface, while an electrostatic repulsion is expected to act between pAA and the magnetite surface. However, electrostatic repulsions do not occur as the carboxylate groups are known to coordinate with the Fe atoms on the surface, as demonstrated in nature for the C-termini of proteins involved in the biomineralization of magnetite in magnetotactic bacteria.

At t₀ (time zero), the solution is composed of only thin hexagonal platelets consistent with other reports about green rust type 2 (Figure 1f and Figure S2; see Supporting Information section 2 for details).

A majority of these green rust platelets are organized in dense star-like aggregates (Figure 1d) that in turn are arranged in strings (Figure 1f). However, as the reaction proceeds, the frequency of dense star-like aggregates decreases while the frequency of open networks of precursor platelets increases (Figure 1e). The formation of these star-like green rust aggregates could be due to the bridging flocculation phenomenon driven by the adsorption of pAA onto the green rust precursor surface (Supporting Information section 3). While magnetite is formed, the green rust crystals are consumed as observed via cryo-TEM where their shape starts to deviate from the ideal hexagon (inset in Figure 1b and Figure S3c). At the end of the reaction, the solution contains only magnetite crystal aggregates, indicating that all of the green rust is completely converted (Figure 1i and Figure S1). At 7 min, magnetite crystals with a flower-like morphology as previously reported have already formed. The Fe$_3$O$_4$ crystals are present either as individual crystals or in chains (Figure 1g and Figures S3 and S4) and have an average size of $\pm 12$ nm, in good agreement with what was previously reported. Surprisingly, a majority of the magnetite crystals always appear to have a crystal size within the range of the calculated average size regardless of the reaction time (see Supporting Information section 4 and Figure S5), while their frequency increased with time. The fact that the crystal size is roughly constant will depend on various factors, such as the formation rate and adsorption of the polymer on the magnetite surface. The SAED pattern of one aggregate shows a single-crystal diffraction pattern (inset in Figure 1c and Figures S1 and S4) indicating the crystallographic alignment of the subunits. Such mesocrystalline structure was previously observed for similar magnetite aggregates synthesized in the presence of pAA, diethylene glycol, and polyarginine. Electron diffraction has confirmed the single-crystalline structure of the Fe$_3$O$_4$ but fails to provide information about the 3D crystallographic organization.

Hence, to fully understand the hierarchical structure of these aggregates and to determine whether the subunits are crystallographically aligned into a mesocrystalline structure, we need to investigate the 3D morphological and crystallo-
graphic organization of the aggregates. For this, we performed electron tomography (Figure 2; see Materials and Methods and Supporting Information section 5) by acquiring a series of high-resolution images at different tilt angles (tomographic tilt series). The point resolution is defined as the first zero of the contrast transfer function (CTF). 46 For our acquisition conditions (0.095 nm/pixel and −300 nm defocus), it is 0.8 nm, while the information limit is ∼1.5 Å (Supporting Information section 5.1). Thus, some images of the acquired tomographic tilt series contain lattice information allowing us to detect the common d spacings for magnetite. To study the orientation of the subunits, the tomographic tilted series was aligned, filtered to point resolution, and reconstructed (Supporting Information section 5.2). The reconstructed tomogram (Figure 2a and Video S1) shows that the structure is composed of subunits and low-density areas (or gaps) that penetrate through the entire volume of the crystal, confirming the cryo-TEM observations. Also, these gaps between the subunits were not uniformly distributed in the structure but with subunits that appear to have partially coalesced. Because the carboxylic acid groups of pAA are known to strongly interact with the Fe ions on the surface of Fe₃O₄,27 these gaps contain most probably the polymer that was adsorbed on subunits during the formation process, in agreement with what was previously observed by elemental analysis of magnetite mesocrystals formed in the presence of polyarginine. 45

To resolve the complexity of the structure and the distribution of the gaps, we have processed the reconstructed tomogram using a multistep algorithm (Videos S2 and S3, and see Supporting Information section 5.3 for more details) to obtain a volume segmentation of the aggregate subunits (Figure 2b,c and Video S4). The processing provides extra information about the shape, the organization of the subunits, and a quantitative estimation of the size range of the subunits. It should be noted that the volume segmentation of the subunits was performed on a cropped volume of the reconstructed tomogram containing a single aggregate to reduce the amount of data to be processed. From the results of the volume segmentation, we determined the diameter range by considering a sphere of equivalent volume representing the segmented subunits. The obtained average diameter of a subunit is 12.1 ± 4.7 nm (Figure 2c), which is twice the size of the smallest feature observed via cryo-TEM (Figure 1b). Therefore, flower-like magnetite crystal aggregates must be formed through a multiple-assembly process. The precursor converts into primary particles that aggregate to form the subunits, which in turn assemble into the flower-like morphology. However, Fe₃O₄ crystal growth promoted by local Fe supersaturation, due to dissolution–reprecipitation of...
that these magnetite aggregates are composed of ∼crystalline plane of magnetite. The results of morphological
dd the experimental might be due to the gold nanoparticles used for labeling of the tomogram. The yellow area in panel a shows the Fourier
a few are on the edge of the magnetically stable single-domain
below the superparamagnetic (SP) regime (<20 nm); however,
at a

The degree of alignment of the subunits can be estimated from
micrograph shows the good level of alignment of the subunits.

The crystallographic orientation of the subdomains was
investigated by a different analysis approach using again the
data of the tomographic tilt series (Figure 3 and Supporting
Information section 5.4). First, a Fast Fourier transform
(FFT) of each image of the tomographic tilt series was
calculated, which contains lattice information in the form of
diffraction spots (Figure 3b). From these spots, the lattice
plane d spacing can be measured. This analysis gives access to
the 3D crystallographic orientation of the subdomains. We
performed Fourier filtering on the FFT images to enhance the
area of the aggregates that have generated the diffraction spot,
a process we term backmapping (Figure 3a; see Supporting
Information section 5.4 for more details). The superimposed
image of the Fourier filtering with the original TEM
micrograph shows the good level of alignment of the subunits.
The degree of alignment of the subunits can be estimated from
the colored area representing the region of the aggregates that
have generated the d spacing in the FFT.

All of the d spacings determined from the FFT tilt series can
be plotted as a function of the rotation angle (Figure 3c),
showing the observed crystalline planes. The d spacings are
consistent with magnetite; however, they are all slightly larger
than the reference X-ray diffraction d spacings due to the
different calibration method. Furthermore, the few reflections
at a d spacing of 4.2 Å might be assigned as the (200)
crystalline plane of magnetite. The results of morphological
and crystallographic analysis of the tomography demonstrate that these magnetite aggregates are composed of ∼10 nm
subunits that are crystallographically aligned in the same
directions. However, a small percentage of the subunits (<1%) exhibit only one-dimensional alignment along the (111)
direction. Although some of the subunits coalesced, we conclude that the aggregates consist of crystallographically
aligned subunits, thus having the main characteristic of a mesocrystalline structure.

To investigate the magnetic properties of individual Fe3O4
mesocrystals, we used off-axis electron holography (EH) in a
transmission electron microscope (Figure 4).48,49 EH measures

the phase shift of the electrons that interact with the magnetic
field and the electrostatic potential of the sample with a spatial
resolution close to 8 nm (see Materials and Methods and
Supporting Information section 6 for details).

The phase shifts related to the magnetic flux density (Figure
4a) and the mean inner potential (MIP) (Figure 4b) are
separated from the total phases retrieved from a pair of
holograms, in which the magnetite chain is magnetically
saturated in opposite directions. The studied chain contains
crystals of different sizes, from ∼30 nm, the smallest, to 120

Figure 3. (a) Single electron micrograph extracted from the tomographic tilt series of a typical magnetite aggregate. (b) FFT of panel a showing
diffraction reflections consistent with the magnetite mineral phase. The noncircled spots correspond to ∼4 Å, not consistent with magnetite, and
might be due to the gold nanoparticles used for labeling of the tomogram. The yellow area in panel a shows the Fourier

Figure 4. Off-axis electron holography analysis of a chain of Fe3O4
mesocrystals containing crystals of variable size from 30 to 120 nm.
(a) Magnetic phase contours (0.2 rad spacing) formed from the in-
plane magnetic field contribution to the phase shift overlapped with
the electron hologram of the mesocrystals. The magnetic induction is
indicated using an arrow and according to the color wheel shown. The
two last crystals on the upper side of the chain show a complex
magnetic state. (b) MIP of the mesocrystal chain shows the
inhomogeneity of the intensity distribution within the crystals.
nm, the largest, as shown in Figure 4. The magnetic flux density map (Figure 4a) shows the magnetic field distribution and its strength visualized using contour lines and colors. A majority of the crystals form a stable single magnetic domain; however, the magnetic state of large crystals at the end of the chain suggests a complex magnetic state with curling flux lines forming a vortex-like state. Three small ∼30 nm crystals on the left side of the chain show no magnetic signal. These crystals are close to the superparamagnetic limit and may not have reversed their magnetization during the reversal. The crystal in the chain marked by “B” that does not overlap with others and has a well-defined single magnetic state has been selected and analyzed in greater detail. Its size is measured as 70 nm using a bright field TEM image (not shown). The magnetic phase shift for a spherical 70 nm Fe₃O₄ crystal with saturation magnetization of 0.6 T is 1.52 rad,⁵⁰ which is in good match with the measured phase shift of 1.5 ± 0.2 rad (Figure S7). The same behavior was reported for mesocrystalline magnetite aggregates formed in the presence of polyarginine,⁴⁵ in contrast with another study in which magnetite mesocrystals synthesized in the presence of diethylene glycol exhibit a superparamagnetic (SP) behavior.¹⁸,²³ Thus, the subunits are crystallographically aligned, and while in the SP regime, they form a stable single magnetic domain, facilitating chain formation. Because the easy magnetization direction for magnetite is along the [111] plane, the magnetism might play a role in the crystallographic alignment of the subdomains. The results show a sequential alignment of the magnetite aggregates, crystallographically and magnetically. These alignments can occur sequentially to each other or simultaneously as the SP subdomains possess a certain degree of rotational freedom. However, there are no experimental results that can distinguish between the two mechanisms. The electrostatic potential of the mesocrystals marked by “A” and “B” is also studied using the measured MIP potentials. The size of crystal A was measured on bright field TEM images as 83 nm (crystal B is 70 nm). For spherical particles, assuming a MIP of 17 V and an accelerating voltage of 300 kV, the MIPs at the center of 83 and 70 nm particles relative to its surrounding are predicted to be 9.2 and 7.77 rad, respectively. These values are larger than the experimentally measured values of 7.9 ± 0.15 and 7 ± 0.15 rad, respectively, suggesting that the Fe₃O₄ mesocrystals have a density that is slightly lower than that of pure magnetite. The discrepancy in the phase shift measurements relative to pure Fe₃O₄ can be explained by the presence of pAA in the gaps of individual units as the electron tomography experiments revealed (Figure 2).

CONCLUSIONS

Here, we present a study of the intermediate stages during the aqueous formation of Fe₃O₄ aggregates in the presence of pAA. The cryo-TEM micrographs (Figure 1 and Supporting Information section 1) reveal that magnetite is formed through a multistep process. First, green rust platelets are formed that transform into ∼5 nm primary particles (Figure 1b). In a second step, the agglomeration of primary particles, probably through an oriented attachment mechanism,³¹,⁵¹ forms magnetite crystals in the size range of 10 nm that represent the subunits. Finally, the aggregation of these magnetite subunits produces the final mesocrystalline magnetic structure. Figure 5 shows the schematic of this proposed stepwise growth mechanism summarizing the experimental observations discussed in this study.

The polymer plays a crucial role in this process. In the early stages of the reaction, green rust platelets aggregate (star-like aggregates) due to the electrostatic absorption of pAA on the platelet surfaces according to the bridging floculation phenomenon. While the green rust is consumed during the formation of magnetite, the colloidal stabilization of the precursor prevails in the floculation with a consequent decrease in the frequency of the star-like aggregates. At the same time, the polymer is also interacting with the forming magnetite mineral phase. This interaction is due to the fact that some of the magnetite crystalline planes expose Fe that can coordinate with carboxylate groups. In fact, the 3D reconstructed volume visualization (Figure 2d,e) of the crystal structure shows that the cracks, propagating through the structure, are located at approximately 90° to each other,
supporting the idea of a directional absorption of the polymer. Therefore, pAA would not absorb completely on the Fe₃O₄ surface giving rise to the depletion interaction 52 (see Supporting Information section 3) that could contribute to promoting the aggregation of both primary particles and subunits.

3D reconstruction of the mesocrystals shows that the subunits are crystallographically aligned in the Fe₃O₄ aggregates. Interestingly, even though the crystals are composed of subdomains in the SP regime, the overall structure displays magnetic properties depending on the final crystal size (either SSD or MD) as expected for magnetite crystals formed in the absence of any additivies. Furthermore, the presence of isolated Fe₃O₄ aggregates at early stages (7 min in Figure 1g) but not in the end product suggests that the magnetism of the crystals also evolves during the reaction.

The magnetic imaging studies reveal that the aggregates arranged in chains present a single stable magnetic domain or a complex state (e.g., vortex) depending on the crystal size. In solution, magnetite aggregates that are not arranged with other magnetite crystals are observed, possibly due to the large size that decreased their diffusion coefficient in solution. Nevertheless, these large isolated aggregates still present a crystallographic alignment (Figures S1 and S4).

This work provides new insights into the formation mechanism of multicore magnetite aggregates that can be useful for designing material for future applications. Controlling the formation step of the subunits or affecting the aggregation step of the magnetite subunit, the shape and magnetic state of the crystals will be helpful for technological applications and/or biomedical applications, such as cancer therapy.

MATERIALS AND METHODS

Magnetite Synthesis. Polyacrylic acid (pAA, Mₙ ~ 450000 Da), FeCl₃·6H₂O, and Fe₂(SO₄)₃·7H₂O were purchased from Sigma-Aldrich. KNO₃ and KOH were purchased from Merck. All chemicals were used as received. All solutions were prepared using Milli-Q water (18.2 MΩ, Millipore, 20 °C), which was degassed using either a nitrogen gas flow or an argon gas flow for at least 30 min prior use. A mixture containing 7 mL of 0.5 M KOH, 10 mL of 0.5 M KNO₃, and 0.0456 g of pAA was prepared and degassed. This mixture was then added to a three-neck round-bottom flask containing 8 mL of 0.08 M Fe₂(SO₄)₃·7H₂O, and immediately after that, a dark green precipitate was formed, which gradually converted into black magnetite. The entire synthesis was performed under a continuous nitrogen flow at 90 °C, with a final pH value of ~13–14. After 4–6 h, the reaction was stopped and the solution product stored in a vial sealed under nitrogen.

Transmission Electron Microscopy. Cryo-TEM. For cryo-TEM, we used 200 mesh Cu grids with Quantifoil R 2/2 holey carbon films (Quantifoil Micro Tools GmbH). For the cryogenic sample preparation, we used an automated vitrification robot (FEI Vitrobot Mark III) to plunge each sample into liquid ethane in preparation for cryo-TEM. 53 Prior to being used, the TEM grids were glow discharged for 40 s with a Cressington 208 carbon coater to render them hydrophilic. Samples were analyzed on the Titan Krios instrument (FEI) operated at 300 kV, equipped with a field emission gun (FEG), a postcolumn GIF, and a post-GIF Gatan CCD camera (2048 × 2048 pixels). A Gatan Digital Micrograph (including DifTools) and MATLAB were used to analyze the TEM image and SAED pattern, respectively. For the acquisition of the tomographic tilt series, a single rotation axis was used to tilt the stage from −68° to 68° in 2° intervals. Focus was determined from the image shift of the gold fiducials, applying beam tilting of 16 mrad between the images. The tomographic tilt series was processed to obtain the reconstruction by using a simultaneous iterative reconstruction technique (SIRT). 54

Off-Axis Electron Holography. The samples were prepared on lacey carbon-covered Cu grids by applying ~50 μL of sample solutions and drying in vacuum. The electron holograms were recorded using the spherical-aberration-corrected FEI Titan “Holo” microscope at Ernst Ruska-Centre for Microscopy and Spectroscopy with Electrons in Forschungszentrum Jülich. It was operated under magnetic field free conditions (Lorentz mode) at an acceleration voltage of 300 kV. The holograms were recorded using a conventional CCD camera at 2048 × 2048 pixels (Gatan Ultrascan) and a Fischione dual-axis tomography holder. Further details regarding the acquisition procedure can be found in Supporting Information section 6.

Crystal Size Measurements. Crystal size distributions were determined by manually measuring the long and short axes of more than 100 individual crystals per sample in TEM images using a MATLAB program. The average of long and short axis for each crystal was taken as the crystal size, and ± indicates the standard deviation of the mean.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.9b01836.

Video of the reconstructed tomogram of a typical magnetite aggregate (AVI)

Additional discussion and supplementary figures (PDF)

Video of the manual segmentation of the aggregate and the gaps (AVI)

Video of the distance transform map obtained from the distance-based watershed process (AVI)

Video of the final labeled segmentation volume (AVI)

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N.A.J.M.S., H.F., G.M., and A. Keizer conceived and designed the experiments. A. Keizer and G.M. performed the synthetic
experiments. H.F., P.H.H.B., G.M., and A. Keizer conceived and designed the TEM, ET, and cryo-TEM experiments. A. Keizer, G.M., and P.H.H.B. performed the TEM, ET, and cryo-TEM acquisition. G.M. and A. Keizer performed the cryo-TEM analysis. H.F. and A. Keizer performed the ET analysis. R.E.D.-B. and A. Kovács conceived and designed the EH experiments. A. Kovács, G.M., and A. Keizer performed the EH acquisition. A. Kovács performed the EH analysis. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**
The authors declare no competing financial interest.

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