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Magnetotactic Bacteria using Graphene Liquid Cell –
Transmission Electron Microscopy**

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Understanding the biomineralization pathways in living biological species is a grand challenge owing to the difficulties in monitoring the mineralization process at sub-nanometer scales. Here, we monitored the nucleation and growth of magnetosome nanoparticles in bacteria and in real time using a transmission electron microscope (TEM). To enable biomineralization within the bacteria, we subcultured magnetotactic bacteria grown in iron-depleted medium and then mixed them with iron-rich medium within graphene liquid cells (GLCs) right before imaging the bacteria under the microscope. Using *in situ* electron energy loss spectroscopy (EELS), the oxidation state of iron in the biomineralized magnetosome was analysed to be magnetite with trace amount of hematite. The increase of mass density of biomineralized magnetosomes as a function of incubation time indicated that the bacteria maintained their functionality during the *in situ* TEM imaging. Our results underpin that GLCs enables a new platform to observe biomineralization events in living biological species at unprecedented spatial resolution. Understanding the biomineralization processes in living organisms facilitate the design of biomimetic materials, and will enable a paradigm shift in understanding the evolution of biological species.

Introduction

Biomineralization is the formation of minerals in living organisms.¹ Properties of these biominerals are suitable for major medical and physical applications, which have motivated researches to biomimic these minerals.² Biomineralization has been reported to occur during the nucleation and growth of crystals, for instance, in ferritins,³ calcium carbonates,⁴ calcium phosphates⁵ and magnetosomes.⁶ Magnetosomes in the fully mature stage are magnetic magnetites, Fe₃O₄.¹ Magnetotactic bacteria are known to biomineralize magnetosomes. Due to the presence of these *in vivo* biomineralized magnetic particles in the cytoplasm, these bacteria align with respect to the geomagnetic field on Earth.¹ Magnetic nanoparticles are very important for their potential uses in both medical and physical sciences. Specifically, they can be used in medical sciences as drug delivery agents,⁷ contrast enhancing tools in magnetic resonance imaging (MRI),⁸ and biomarkers for viruses,⁹ bacteria¹⁰ and cancer¹¹ and furthermore, in physical sciences for ferrofluids,¹² high density data storage¹³ and spintronics.¹⁴ Since the yield of *in vivo* biomineralized bacterial magnetosome is low for industrial applications, *in vitro* synthesis methods have to be developed to result in higher magnetosome yield while reaching the same material properties of *in vivo* biomineralized magnetosomes, such as chemical composition, magnetism and uniform size.¹⁵ This

makes understanding the *in vivo* magnetosome biomineralization of paramount importance.

To understand magnetosome biomineralization, several groups reported subculturing of iron deplete bacteria with iron replete medium, and observed the nucleation and growth of magnetosomes via time-resolved conventional transmission electron microscopy (TEM) imaging of the newly formed magnetosomes.^{6,16,17} Baumgartner *et al.* reported that magnetite formation happens through the formation of highly disordered phosphate-rich ferric hydroxide, then its conversion into ferric (oxyhydr)oxide, and finally the formation of magnetite.¹⁶ Staniland *et al.* described the formation of transient hematite and was able to observe magnetite formation after 15 minutes of iron induction through TEM and X-ray absorption spectroscopy (XAS) analyses.¹⁷ Using time-resolved imaging and chemical analysis in TEM, Firlar *et al.*⁶ reported the presence of amorphous ferric hydroxide when magnetosomes were smaller than 10 nm, followed by partial crystallization with 2 line ferrihydrite in particles with diameter in the range of 10-15 nm. For particles with sizes larger than 15 nm, fully magnetite phase was detected. Both Staniland *et al.* and Firlar *et al.* reported the formation of hematite around magnetite as well.^{6,17} All of these works were carried out by drying the bacteria after the desired stage of biomineralization was reached, thus imaging was executed on the dead bacteria.

In order to observe the biomineralization kinetics in living species, the bacterial culture needs to be preserved in the growth medium. Unless preserved cryogenically or as hermetically sealed via liquid cells, bacteria will be dehydrated in the TEM column, losing their native properties. Liquid-cell TEM enclosures using Si₃N₄ membranes¹⁸ and graphene monolayers¹⁹ have been introduced to enable the visualization of hydrated dose sensitive samples and allow monitoring of sample specific dynamics, which is not possible with cryofixation of the samples. Si₃N₄ membrane fluid cells involve

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encapsulation of samples between two electron transparent Si_3N_4 membranes.^{20,21} This technology also enables delivering liquid to the liquid cell chamber during TEM imaging, allowing to monitor dynamics relevant to reactions taking place due to the interaction of the host sample in the enclosure with the external fluid flow.^{19,22–24} This technique was also employed by Woehl *et al.* to prove that bacteria could remain viable in TEM.²⁵ However, Si_3N_4 membranes are relatively thick (15–50 nm), and due to extra bowing in vacuum, the spatial resolution will be reduced significantly. In addition, their microfabrication technology is costly and difficult.²⁶ Graphene liquid cells (GLC), on the other hand, provide hermetic seal to the samples via wrapping them with two monolayers of graphene with the sample inside.²⁷ Graphene is very strong, biocompatible and electron transparent.^{28–31} Furthermore, graphene scavenges reactive radicals, which form via the radiolysis during electron-sample interaction.³² Whole cell imaging ability of GLCs has been reported by Park *et al.* by resolving viruses and cytoskeleton structure of cells.³³ *Bacillus subtilis* was imaged using GLC-TEM by Mohanty *et al.*¹⁹ Furthermore, crystal structure and chemistry of ferritin proteins were studied by Wang *et al.*³⁴ In addition, by controlling the electron beam dose, it was shown that the formation of H_2 and other reactants can be controlled in GLCs.^{34,35}

Currently, the abilities of both keeping the cells viable and having enough resolution to resolve biomineralization events in sub-nm scale concurrently are missing. Therefore, in this study we employed the GLC-TEM imaging technique to monitor the magnetosome biomineralization in magnetotactic bacteria. To achieve this, we cultured the bacterium in iron deplete growth medium. Once passed through the log-phase, induction of iron was carried out by subculturing these bacteria with iron replete growth medium. At this stage, Fe^{3+} ions are internalized by the bacteria and formation of magnetosomes occur. Right after the initiation of subculturing, an aliquot from the bacteria culture was encapsulated in GLC (Fig. 1a) and the formation and growth of magnetosomes were monitored in real time using TEM (Fig. 1b–d).³⁶ Encapsulation of bacterial culture in graphene and preservation of growth medium surrounding the particle with intact graphene was verified via low loss electron energy spectroscopy (EELS). Characterization of the mature magnetosomes was carried out via iron L_3 core edge EELS and formation of magnetosomes were monitored both qualitatively and quantitatively via line profiles drawn over the magnetosomes in TEM images indicating the mass-contrast progression through biomineralization.

Experimental

Sample Preparation

Magnetospirillum magneticum (ATC 700264) was used during biomineralization experiments. ATCC Medium 1653 revised magnetic spirillum growth medium (MSGM) with iron deplete medium was used to subculture them under microaerobic conditions. After the subculturing of the bacteria, for the induction experiment, ferric quinate added iron replete MSGM was used. Right after induction, GLC preparation was carried out.

TEM Imaging

1 μl sample was drop cast on 2000 mesh graphene coated copper grid (Graphene Supermarket, Graphene Laboratories Inc.) and the

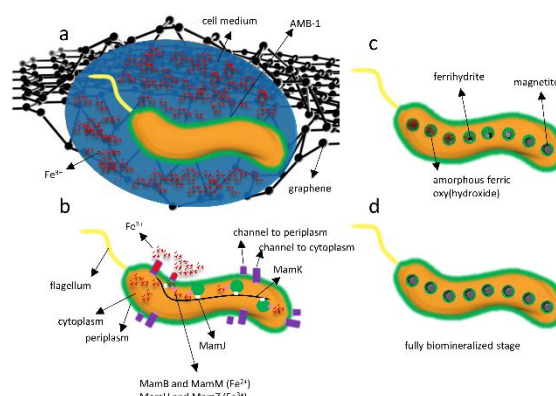


Fig. 1 (a) Schematic showing in vivo bacterial magnetosome biomineralization phenomenon in a GLC. (b) Endocytosis of iron ions (red color) through two separate channels: one into the periplasm (purple color-small) and then to the invaginated vesicles,^{37,38} and the other to the cytoplasm (purple color-large), which later on goes into the vesicle through iron channels. MamB and MamM proteins control transport of Fe^{2+} and MamH and MamZ proteins control the transport of Fe^{3+} from the cytoplasm into the vesicles (pink color).³⁷ MamK is the protein forming filament (black color). MamJ protein controls attachment of particles onto the chain (white color). (c) Formation of iron oxides in the bacterium. Oxygen in the water based cell medium diffuses into the bacterium and causes iron oxide mineral formation.³⁹ Progression is illustrated as the particle growth from left to right in the bacterium. Mms6 proteins control the formation of magnetite crystal in the presence of iron ions (blue color). (d) Fully grown particles in each vesicle are shown in the final state of biomineralization.

secondary graphene coated copper grid was added onto it forming the graphene encapsulation. TEM imaging was carried out at 80 kV using JEOL 1220 and Hitachi HT7700. Image acquisition was carried out via Digital Micrograph. During video recording and image acquisition, 0.1 (200 e/nm^2 per frame) and 1 second exposures (2000 e/nm^2 per frame) were used, respectively.

HAADF-STEM EELS

EELS analysis was carried out using Hitachi HD 2300 STEM with Gatan Enfina EELS detector. 3 mm EELS aperture and 0.1 eV/channel energy resolution were used. 159 e/nm^2 (3×10^{-6} sec beam exposure) and 1.11 $\times 10^9$ e/nm^2 (21 sec beam exposure) were used for low loss and Fe L_3 edges, respectively. For the L_3 edge, background removal was carried out with a 30eV window in Digital Micrograph. 3 channel spectrum smoothing and reference spectra fitting to experimental Fe L_3 spectra was carried out in OriginPro 2016. For the analysis of the oxidation state of iron, Fe^{2+} (octahedral), Fe^{3+} (tetrahedral), Fe^{3+} (octahedral) and $\text{FeO}(\text{OH})$ were fitted to the spectra and ratio of Fe^{2+} (octahedral)/ Fe^{3+} (tetrahedral)+ Fe^{3+} (octahedral) was computed for the determination of the iron oxide type.

Fluorescence Imaging

SYTO 9 and propidium iodide (Fisher Scientific Company LLC, Life Technologies L7007) was used as fluorescence stains for the

investigation of bacterial viability. 0.5 μl bacteria was dropcast on glass slide and cover slip was added onto it. It is also sealed by nail polish to eliminate air flow into the medium. Fluorescence imaging was carried out using Olympus BX-51.

Results and discussion

Comparison of conventional and GLC-TEM imaging of magnetotactic bacteria are reported in Fig. 2a and b, respectively. Higher image contrast was reported in the conventionally imaged bacterium showing crisper details of the magnetosomes, but the bacterium in that stage is assumed to be dead due to the nature of the drop casting TEM sample preparation. Specifically, sample was let dry on the bench-top and then placed in the TEM chamber, both of which eliminates all liquid content. Because the bacterium lacks liquid content, it becomes more tolerant to electron beam induced damage. On the other hand, the bacterium in GLC enclosure is more resistant to electron beam induced radiation damage than liquid cells with Si_3N_4 membranes.^{32,34,35,40} This is because of the less thickness of both the support and the surrounding liquid, causing accumulation of less electron beam induced energy, and thus less formation of radiation by products upon electron beam exposure. Furthermore, thicker membranes, under the same electron dose and voltage cause higher radical yield and higher rate of secondary radical reactions.⁴¹ When electron overexposure is applied, the formation of hydrogen molecule bubbles is observed,³⁵ which is also an indication that the sample is kept in liquid environment. In this work, approximately 2000 e/nm^2 and 200 e/nm^2 was used for image acquisition and video recording, respectively, which are comparable to the electron dose used for cryo-TEM imaging.⁴²

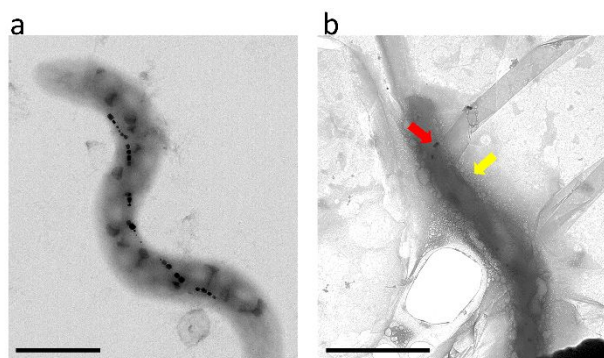
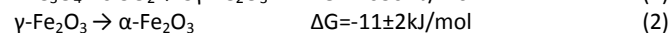
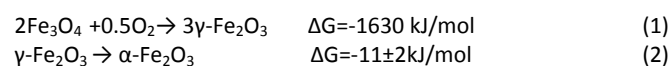


Fig. 2 Comparison of BF-TEM images of magnetotactic bacteria, which underwent biomineralization in conventional imaging and GLC imaging methods. (a) Bacteria in growth medium was drop cast on continuous carbon grid and left to dry for 5 minutes before loading to the TEM holder. Scale bar: 500 nm. (b) Bacteria was encapsulated in GLC and directly loaded into the TEM holder. Red arrows show magnetosomes and the yellow arrow in GLC shows the presence of

hydrogen molecule bubbles formed via electron over exposure. Scale bar: 1000 μm .

Verification of the intact graphene with entrapped water was done by carrying out low loss EELS. This analysis shows the presence of graphene optical gap at 6 eV, water exciton peak at 8.5 eV and graphene $\sigma+\pi$ bond at 14 eV, as shown in Figure 3a. These are all indications of the proper encapsulation of the bacterium in between graphene with the growth medium surrounding it, in addition to the visual confirmation reported in Figure 2b. Further investigation of the chemistry in the mature magnetosomes was executed by iron L_3 core edge EELS analysis as shown in Figure 3b. The collected spectrum was fitted with reference spectra of Fe^{2+} (octahedral), Fe^{3+} (tetrahedral), Fe^{3+} (octahedral) and $\text{FeO}(\text{OH})$. The relative ratio of Fe^{2+} to Fe^{3+} helps to investigate the final structure of the magnetosomes when kept properly in the liquid growth medium. This ratio is calculated to be 0.35. The fact that this ratio is 0.5 for a perfect Fe_3O_4 ($[\text{Fe}^{2+}]_{\text{tet}}[\text{Fe}^{2+}, \text{Fe}^{3+}]_{\text{oct}}$),⁴³ the value of $\text{Fe}^{2+}/\text{Fe}^{3+}=0.35$ suggests two possible contributions of additional Fe^{3+} to this ratio: (i) Presence of another iron oxide, which contributes to a higher percentage of Fe^{3+} ; (ii) Little amount of Fe^{3+} is present in the extracellular region of bacterium. Specifically, 2 ml ferric quinate (0.01 M, that is, 0.27 g FeCl_3 and 0.17 g quinic acid in 100 ml H_2O) was added into 1 liter of growth medium to obtain the iron rich growth medium. During iron induction, 18 ml bacteria culture was subcultured with 6 ml iron rich growth medium. Furthermore, the tightly packing nature of graphene encapsulation due to Van der Waals forces suggests that the medium surrounding the bacterium is thin. Therefore, contribution of Fe^{3+} in the extracellular region to the Fe^{3+} EELS L_3 signal obtained from individual magnetosome is considered negligible. As suggested earlier by Firlar *et al.*⁶ through Gibbs free energy calculations, magnetite may have been oxidized to hematite, supporting the first possibility. This occurs first by the conversion of magnetite to maghemite and furthermore to hematite spontaneously, which may have caused the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio to deviate from 0.5. ΔH_f° for magnetite, maghemite, hematite and oxygen are -1118.4,⁴⁴ -809.0,⁴⁴ -824.2⁴⁴ and 0⁴⁵ kJ/mol, respectively, and S_{298}° for magnetite, maghemite, hematite and oxygen are 146.4,⁴⁴ 102.4,⁴⁴ 87.4⁴⁴ and 205.0⁴⁵ J/(mol \times $^\circ\text{K}$), respectively. The conversion of magnetite to maghemite and furthermore to hematite are defined by eqn (1) and eqn (2),



, showing the spontaneous conversion of magnetite to maghemite and hematite. Even though graphene liquid cells reduce the amount of beam induced radicals forming in solution, one should further study the effect of oxidizing radicals, oxygen gas, and reducing radicals formations during imaging. It is possible these radicals could change the oxidation state of iron during EELS spectrum acquisition."

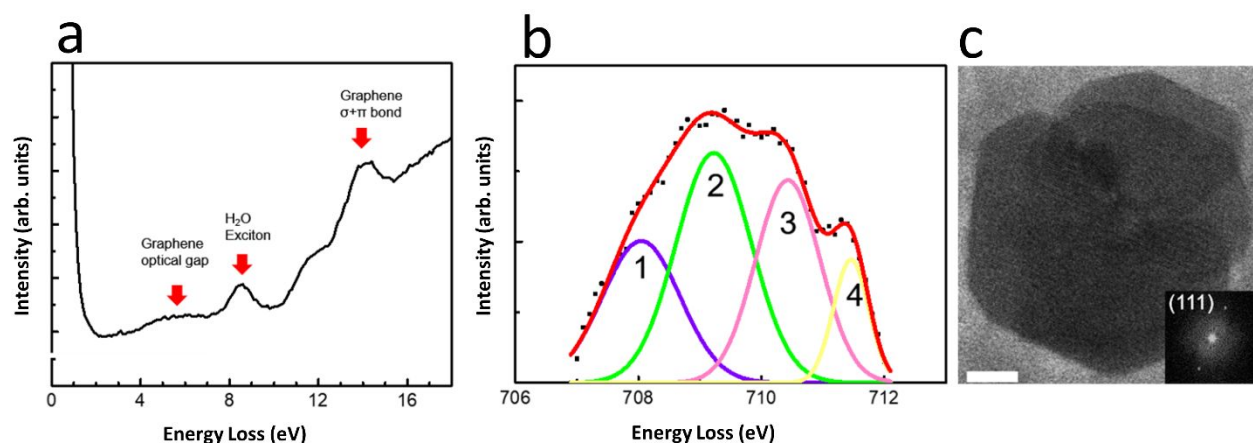


Fig. 3 (a) Low loss EELS collected at close proximity of magnetotactic bacterium encapsulated within graphene liquid cell. Fingerprints for graphene and water show proper bacterial sample preservation in between layers of graphene. (b) Fe L_3 core EEL spectrum collected from mature magnetosomes. The ratio of the areas between peak 1 to peaks (2 + 3), corresponding to Fe $^{2+}$ (octahedral) to Fe $^{3+}$ (tetrahedral + octahedral) ratio, is 0.35. Peak 4 is attributed to FeO(OH). (c) BF-STEM image collected from three overlapping magnetosomes showing Fe $_3$ O $_4$ (111) interplanar spacing in the FFT collected (inset). Scale bar: 10 nm

On a separate magnetosome, the bright field STEM image collected showed lattice fringes referring to magnetite (111) through fast Fourier transformation (FFT) analysis (Fig. 3c). Furthermore, this shows that not all the magnetosomes have the hematite layer as discussed above.

In order to achieve time resolved monitoring of magnetosome nucleation and growth, iron replete growth medium was mixed with the bacteria in iron deplete growth medium ($t=0$ sec). The bacteria in growth medium was sandwiched between monolayers of graphene to form GLC, and the first image of the bacterium was recorded at $t=70$ minutes after induction and is reported Fig. 4a. Red arrow indicates the MamK which is actin-like protein. As given in Uebe and Schuler,³⁷ with the polymerization of MamK, filament form and with the interaction of this filament and MamJ on the magnetosome, magnetosomes are aligned into chains. Residual magnetosomes were reported to be present due to the small quantity iron present in the iron deplete medium. *In vivo* biomineralization was observed by the sequential imaging. Specifically, the image recorded at $t=115$ minutes is reported in Fig. 4b. Nucleation and growth of the two magnetosomes with sizes around 4 nm are observed, as marked with the blue and green arrows. More than 70% bacterial viability was reported using fluorescence imaging before the induction was carried out. De Jonge and Peckys⁴⁶ indicated that even though fluorescence imaging via propidium iodide (red) and SYTO 9 (green) is considered the proper way for cell viability measurement, cells could be dead even due to electron-bacteria interaction without the deterioration of the cell wall, which would prevent the entry of the red dye into the bacteria. A more reliable way to measure the cell viability suggested by the same group was to monitor the cellular functions during *in situ* TEM, which they reported to be not feasible. As a response to that work, Kennedy later showed the expression of LuxI-LVA after the induction of *E. Coli* with isopropyl β -D-1-thiogalactopyranoside (IPTG) during *in situ* STEM.⁴⁷ Very

recently, Firlar *et al.* also monitored live insulin secretion from pancreatic beta cells using GLC-TEM.⁴⁸ In addition to fluorescence imaging for viability examination, they also reported insulin secretion and exocytosis which only occur when the pancreatic islet cells are viable. In this work, the *in vivo* biomineralization, which only happens when the bacteria are alive, indicates that these bacteria were still viable during GLC-TEM imaging when exposed to 200 e/nm 2 per frame during video recording and 2000e/nm 2 during image acquisition.

TEM images in Fig. 5a and c were taken after 17 minutes of iron induction and shows a bacterium wrapped in GLC. As discussed earlier, the surrounding bubbles indicate the formation of H $_2$ molecules in growth medium due to the electron induced radiolysis. TEM images in Fig. 5b and d show the bacterium image after 31 minutes of iron induction. An increase in the image contrast is visible by comparing the magnetosome image intensity in Fig. 5c and d, which was further quantified by the line

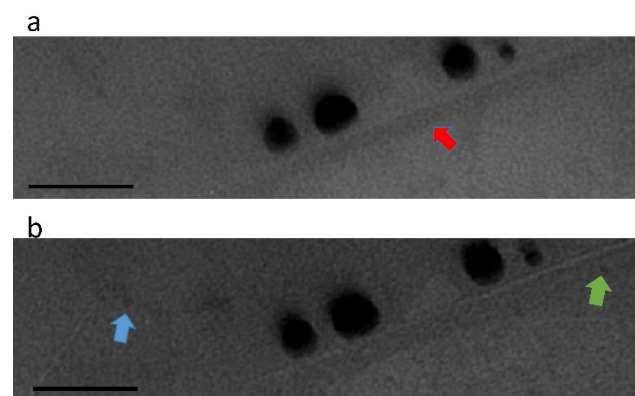


Fig. 4 TEM images of magnetotactic bacterium grown in iron depleted growth medium, mixed with iron replete growth medium, and encapsulated in GLC. Red arrow shows MamK. Blue and green arrows indicate formation of magnetosomes. (a) 70 min after GLC encapsulation and (b) 115 min after GLC encapsulation. Scale bar: 50 nm.

profile comparison across the magnetosome at two time stamps in Fig. 5e. The green rectangles drawn on the profiles show the contrast increase for the magnetosome imaged after 31 minutes of induction. This indicates that the mass density of magnetosomes increases pointing to an increase in the formation of Fe_3O_4 phase. This can further be elaborated with the image contrast analysis using eqn (3).

$$C = \frac{\Delta I}{I_b} = \frac{I_s - I_b}{I_b} = \frac{\Delta t * N_0 * \sigma * \rho}{A} \quad (3)$$

where C , I_b , I_s , Δt , N_0 , σ , ρ and A are contrast, background image intensity, feature image intensity, thickness difference, Avogadro's number, elastic scattering cross section, density and molecular weight, respectively. Using eqn (3) for the particles in Fig. 5c and d, the contrast increases in the 14 minute course of biomineralization, this in turn resulted in thicknesses, calculated using eqn (4),

$$\Delta t = \frac{A * C}{N_0 * \sigma * \rho} \quad (4)$$

Alternatively, this change in image contrast could be due to the increase in mass density of the particle over time, which is known

to progress from 4.2^{49} (ferric oxyhydroxide) to 5.2^{50} (magnetite) g/cm^3 . For the complex nature of the analysis, we have not taken into account the particle rotation and diffraction contrast effects, which may have further affected the observed contrast change. So, the increase in image contrast in the magnetosome during TEM imaging as a function of incubation time indicates progression of the biomineralization event either through an increase in thickness or an increase in density.

Several models were put forward for the elucidation of magnetosome biomineralization. Schuler *et al.*⁵¹ claimed that Fe^{3+} is internalized and reduces to Fe^{2+} . Then, Fe^{2+} reoxidizes to first form low density hydrous oxide, and then to high density ferrihydrite. Finally, one third of Fe^{3+} is converted to Fe^{2+} to form magnetite. Jogler and Schuler⁵² stated that iron is internalized and due to its supersaturation in the cytoplasm, it is encapsulated into the already formed magnetosome vesicles by MamB and MamM proteins, or iron is sent from the periplasm to the vesicles directly by the same proteins. Partial reoxidization of iron caused by MamT causes the formation of high activity ferric oxide, which may further react with dissolved Fe^{2+} forming magnetite. Arakaki *et al.*³⁶ suggested that magnetosome membrane invaginations from the cytoplasmic membrane occur first, with the arrangement of these along with cytoskeletal filaments, and then

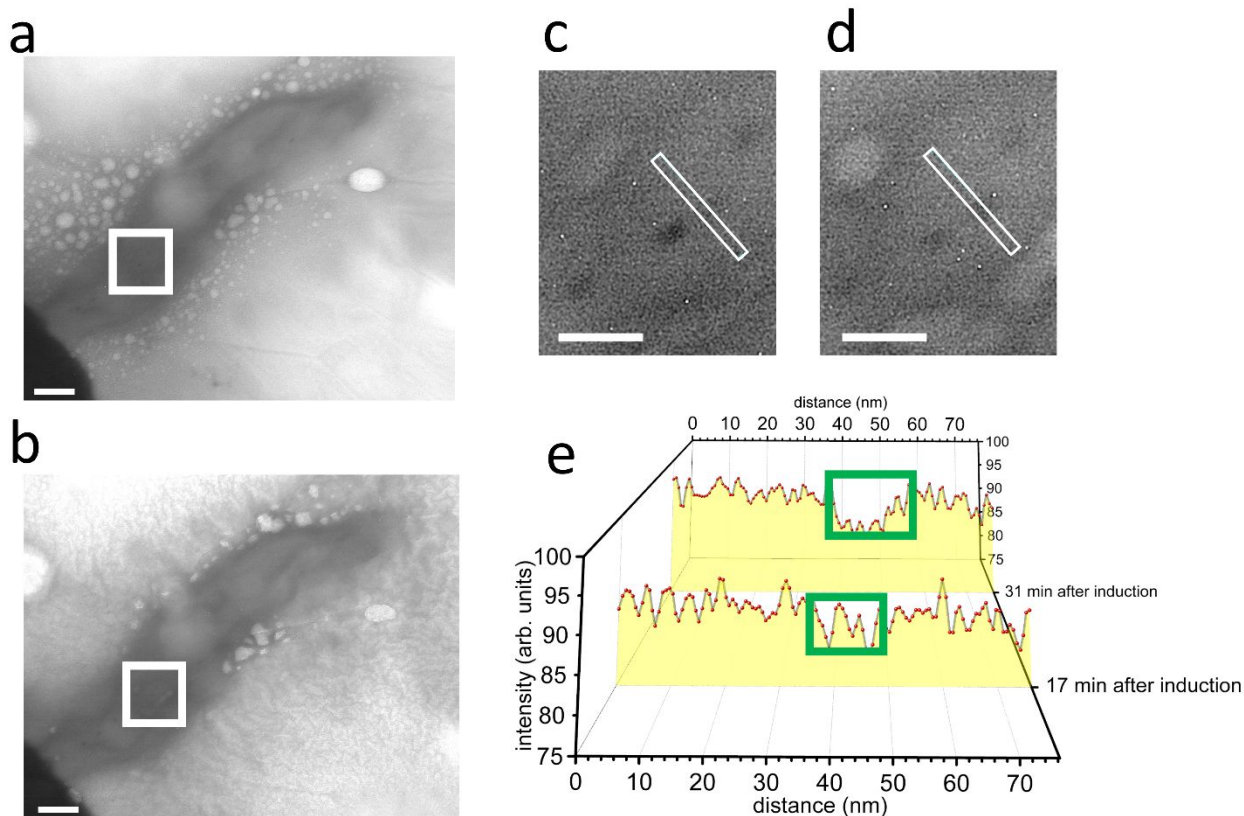


Fig. 5 TEM images showing an increase in image contrast throughout the biomineralization event in the same bacterium encapsulated within GLC. (a) $t=17$ minutes after induction, and (b) $t=31$ minutes after induction. Scale bar: 200 nm. Zoomed in images with the line profiles drawn across individual magnetosomes are shown in (c) for the marked square in (a), and (d) for the marked square in (b). Scale bar: 50 nm (e) The comparison of image contrast evolution in magnetosomes. Higher contrast for the magnetosome imaged after 31 minutes induction indicates the progress of biomineralization event.

external iron is transported into the vesicles by transmembrane proteins and magnetite crystal formation by magnetosome proteins.

Conclusions

In this work, we demonstrated the *in vivo* magnetosome biomineralization events in magnetotactic bacteria in liquid environment using GLC-TEM imaging. Usage of graphene layers instead of two relatively thicker Si₃N₄ membranes in fluid cell TEM holder enabled us to resolve sub 10 nm magnetosomes. To initiate the biomineralization, bacteria grown in iron deplete medium was subcultured with iron replete growth medium and wrapped in between graphene monolayers. The intactness of fingerprints for graphene and water in low loss EELS, respectively. The final chemical composition and crystal structure of biomineralized magnetosome were characterized via the analysis of Fe L₃ edge and bright field STEM imaging, respectively, and found to be magnetite with some magnetosomes having a trace amount of hematite. The enhancement of the contrast profile in TEM images indicated the progression of biomineralization due to the accumulation of more magnetite in the magnetosomes increasing the mass-thickness contrast. Our finding on the progress of the biomineralization process is a good indication that the bacteria were viable and maintained their cellular activities during TEM imaging. To the best of our knowledge, this is the first study showing the applicability of GLCs for *in vivo* biomineralization events in TEM. These results are of utmost importance to the biomaterials and biological community as our work introduces a new platform to investigate the *in vivo* biomineralization in living biological organisms.

Conflicts of interest

There are no conflicts to declare

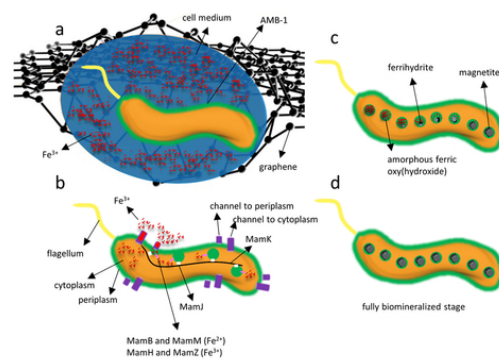
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Magnetosome biomineralization in magnetotactic bacteria was observed by keeping the bacteria alive using graphene liquid cell transmission electron microscopy.

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