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Controlled formation of magnetite crystal by partial oxidation of ferrous hydroxide in the presence of recombinant magnetotactic bacterial protein Mms6

Yosuke Amemiya[a], Atsushi Arakaki[a], Sarah S. Staniland[b], Tsuyoshi Tanaka[a], Tadashi Matsunaga[a,*]

[a]*Department of Biotechnology, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho Koganei, Tokyo 184-8588, Japan*
[b]*Institute of Cell Biology, University of Edinburgh, Edinburgh EH9 3JR, UK*

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Abstract

Mms6 is a small acidic protein that is tightly associated with bacterial magnetite in *Magnetospirillum magneticum* AMB-1. This protein has previously shown iron binding activity, allowing it to generate uniform magnetic crystals by co-precipitation of ferrous and ferric ions. Here, magnetite crystals were formed by the partial oxidation of ferrous hydroxide in the presence and absence of Mms6. The crystals synthesised were systematically characterised according to their sizes and morphologies using high-resolution transmission electron microscopy. Mms6-mediated synthesis of magnetite by this methods produced crystals of a uniform size and narrow size distribution with a cubo-octahedral morphology, similar to bacterial magnetite observed in *M. magneticum* AMB-1. The crystals formed in the absence of Mms6 were octahedral, larger with an increased size distribution. Protein quantification analysis of Mms6 in the synthesised particles indicated tight association of this protein onto the crystal. Furthermore, high affinities to iron ions and a highly charged electrostatic quality suggest that the protein acts as a template for the nucleus formation and/or acts as a growth regulator by recognising crystal faces. The method introduced in this study presents an alternative route for controlling the size and shape of magnetite crystals without the use of organic solvent and high temperatures.

Keywords: Magnetite; Nanoparticle; Crystal growth; Biomimetic material; Biomineralisation; Magnetotactic bacteria

1. Introduction

Magnetic iron oxide particles, such as magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3), are widely used in the development of medical and diagnostic applications such as magnetic resonance imaging (MRI) [1], cell separation [2,3], biosensing [4], hyperthermia [5], and site-specific chemotherapy [6]. Procedures for the controlled formation of uniformly sized particles at the nanometer scale have been developed by the thermal decomposition of organo-iron precursors using amphiphilic molecules such as surfactants or fatty acids [7,8]. However, these methods require the use of organic solvents or extremely high temperatures. Given the increasing demand for magnetic nano-materials, alternative processes for the simple formation of size- and shape-controlled iron oxide crystals in aqueous environments are required.

Biomineralisation is an elaborative process that produces uniformly sized, self-assembly nano-structures, often with complex morphologies [9]. Biomineralisation uses organic molecules for the nucleation and subsequent crystal growth processes under relatively mild conditions (at a near-neutral pH and ambient temperature), leading to characteristic crystallographic orientations. The biological and chemical principles of biomineralisation have, therefore, been studied in order to exploit these principles for use in advanced nano-material development [10–13].

With a few exceptions, magnetotactic bacteria produce fine magnetite crystals under ambient conditions. The sizes

*Corresponding author. Tel.: +81 42 388 7020; fax: +81 42 385 7713.
E-mail address: tmatsuna@cc.tuat.ac.jp (T. Matsunaga).

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and morphologies of the nano-sized magnetite crystals are highly consistent within bacterial species or strains [14], suggesting the presence of a biologically controlled mineralisation process at the genetic level. Much attention has been focused on proteins associated with the organic membrane that envelops bacterially produced magnetite in order to analyse these precisely regulated molecular mechanisms [15,16]. Recent proteome analysis of this membrane has identified several novel proteins. The direct roles of these proteins in magnetite biosynthesis have been elucidated by several molecular studies [17–20]. Mms6 represents a class of proteins that are tightly associated with the bacterial magnetite surface in *Magnetospirillum magneticum* AMB-1 [21]. The Mms6 amino acid sequence is amphiphilic, and consists of an N-terminal LG-rich hydrophobic region and a C-terminal hydrophilic region containing multiplets of acidic amino acids. Following competitive iron binding analysis with other inorganic cations, it has been suggested that the C-terminal region is an iron-binding site. Furthermore, magnetite has been formed by co-precipitation of ferrous and ferric ions in the presence of Mms6 producing uniformed crystals with sizes ranging from 20 to 30 nm, while the absence of this protein resulted in the formation of magnetic particles of irregular shapes and sizes [21]. Recently, Prozorov et al. [22] performed the co-precipitation of ferrous and ferric ions in the presence of Mms6, ferritin (that is also iron binding protein) and bovine serum albumin (BSA). They reported that only Mms6 had a size and shape regulating effect on the magnetite crystals. However, the exact role of Mms6 in the magnetite synthesis process still remains unknown.

Here, a partial oxidation of ferrous hydroxide method was employed to synthesise particulate magnetite with and without the addition of Mms6, and their crystallographic characteristics were analysed and compared. Regulatory mechanisms were also investigated by determining the structural behaviour of Mms6 in solution, and the localisation of the protein in synthesised magnetite crystals. This preparation process may provide an alternative method for the simple regulation of nano-sized magnetic particles.

2. Experimental section

2.1. Purification of Mms6 expressed in Escherichia coli

Recombinant Mms6 was expressed as a fusion protein with Glutathione S-transferase (GST) in *Escherichia coli* strain BL21. The recombinant plasmid pGEX 4T-*mms6* which harbours the *mms6* gene was constructed by cloning PCR products into the expression vector pGEX 4T-1. The *mms6* gene was amplified from the AMB-1 genomic DNA as described previously [21]. The *E. coli* BL21 transformant was cultured in Luria-Bertani broth at 37 °C under isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction. The cells were collected by centrifugation, resuspended in STE (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 100 μg/mL PMSF, pH 8.0) buffer and stored at −80 °C until use.

GST-Mms6 was purified using glutathione agarose (GA) beads (GE Healthcare Biosciences, Piscataway, NJ) according to a protocol reported previously [23,24] with minor modifications. *E. coli* was treated with 100 μg/mL lysozyme for 15 min and disrupted by ultrasonication in the presence of 5 mM DTT and 0.75% *N*-laurylsarcosine (Sarkosyl). Following centrifugation, the supernatant was filtered with a syringe filter unit (pore size: 0.20 μm). Triton X-100 (final concentration: 1.5%) and GA beads were added to the supernatant and the suspension was allowed to incubate for 1 h at 4 °C with continuous agitation. The suspension was then centrifuged, the supernatant was discarded and the GA beads were washed with PBS thoroughly. The GA beads were resuspended in PBS, and thrombin (GE Healthcare Biosciences, Piscataway, NJ) was added to the solution for the digestion of GST-Mms6 on the GA beads. The mixture was incubated for more than 16 h at room temperature with continuous agitation. The GA beads were collected by centrifugation and resuspended in a denaturing buffer (6 M guanidine hydrochloride, 50 mM Tris, pH 10) for the elution of proteins from the GA beads. The GA bead suspension was centrifuged and the supernatant was collected. This elution step was repeated. The collected samples from both the elution steps were combined and concentrated using Centricon YM-3 (Milipore Corporation, Bedford, MA). Further purification of Mms6 was conducted by size exclusion chromatography using superdex 75 10/300 GL column (GE Healthcare Biosciences, Piscataway, NJ) and by using a fast protein liquid chromatography (FPLC) system (ÄKTAexplorer 10S, GE Healthcare Biosciences, Piscataway, NJ). The fraction containing Mms6 was concentrated and then diluted in the same volume of refolding buffer (50 mM Tris, 1 mM EDTA, 0.1 M L-arginine and 10% (V/V) glycerol, pH 8.0). The purified protein was dialysed against buffer A (50 mM Tris, 1 mM EDTA, 1 M guanidine hydrochloride and 10% glycerol, pH 8.0), buffer B (50 mM Tris, 1 mM EDTA and 10% glycerol, pH 8.0) and buffer C (10 mM Tris and 10% glycerol, pH 8.0), respectively, for more than 6 h. Finally, the protein solution was dialysed twice against 0.1% NH_4OH solution for at least 6 h per repetition. Each of the dialysis steps were performed at 4 °C. Proteins obtained at each purification step were analysed by Tricine-SDS-PAGE, staining with Coomassie brilliant blue G-250 (Bio-rad, Hercules, CA).

2.2. Characterisation of Mms6 in aqueous solution

The aggregation behaviour of Mms6 in aqueous solution was analysed by fibre-optics dynamic light scattering spectrophotometer equipped with a solid-state laser (100 mW, 532 nm) and a temperature control unit (FDLS-3000, Otsuka Electronics, Osaka). Analysis was performed at 25 °C and a total of 100 runs were examined for one analysis. Ammonium hydroxide solution (0.1%, pH 10) containing 5.8 μg/mL of Mms6 was used for the analysis. One mg/mL BSA in 0.1% NH_4OH was used as a control.

The zeta potential of the purified Mms6 suspension was measured on an ELS-8000 (Otsuka Electronics, Osaka). The protein solution containing 2 mM NaCl was adjusted to pH values within the range 3–8, and left overnight at 5 °C with continuous stirring to allow complete equilibration. BSA was used as a control. Protein solutions used in this examination were adjusted to 0.5 μg/mL, respectively.

The secondary structure of Mms6 was analysed using a circular dichroism (CD) spectrometer (J-720, JASCO, Tokyo). Specimens were scanned from 190 to 250 nm at 25 °C with a 1 nm bandwidth and a scan rate of 20 nm/min. A total of five scans were conducted for one analysis and the background spectrum (0.1% NH_4OH) was subtracted. A protein solution containing 130 μg/mL of Mms6 was used for the analysis.

2.3. Formation of magnetite crystals

Magnetite crystals were formed by partial oxidation of ferrous hydroxide [25–27]. One mL of the reaction solution containing 30 mM $FeSO_4 \cdot 7H_2O$, 100 mM KOH, 400 mM KNO_3 was prepared with or without Mms6 (5.6 μg/mL). The pH of the solution containing 5.6 μg/mL of Mms6 was determined to be approximately 7.5. In order to bind iron ion with Mms6, the mixture was incubated at room temperature for 5 min with continuous nitrogen gas bubbling. The solution was then heated to 90 °C in a water bath for 5 min under nitrogen. The tube was