Crystal orientation analyses of biominerals using Kikuchi patterns in TEM

Kazuko SARUWATARI*, Junji AKAI**, Yoshihiro FUKUMORI***, Noriaki OZAKI****, Hiromichi NAGASAWA**** and Toshihiro KOGURE*

"Department of Earth and Planetary Science, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
"Department of Geology, Faculty of Science, Niigata University, 8050 Ikarashi 2, Niigata 950-2181, Japan
"Department of Life Science, Graduate School of Natural Science, Kanazawa University, Kakuma machi, Kanazawa, Ishikawa 920-1192, Japan
****Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

This paper shows that Kikuchi pattern analysis in a transmission electron microscope (TEM) using recent techniques is superior to conventional electron diffraction analysis for determining crystal orientation in an assemblage of small crystals such as those of biogenic origin. A CCD camera with a wide view and large dynamic range was used together with a dedicated program to analyze the patterns and enable real-time and unique determination of crystal orientation. Convergent illumination of the incident beam was effective in enhancing the Kikuchi patterns and in improving spatial resolution. Two example biominerals are characterized by Kikuchi pattern analysis; one is spinel-law twins in several tens of nanometers width magnetite crystals from magnetotactic bacteria. The other is crystal orientation and the alignment of calcite fragments in coccolith.

Keywords: Kikuchi pattern, TEM, CCD camera, Biomineral, Magnetosome, Coccolith

INTRODUCTION

Biologically controlled mineralization (BCM) generally constructs hierarchical structures composed of organic substances and inorganic crystals called biominerals whose polymorph, size, shape, and orientation are precisely controlled (Mann, 2001). Crystal orientation in biominerals may be regulated by an organic-inorganic interaction during their nucleation on the organic matrices. For this reason, the determination of crystal orientation is important when considering their formation mechanism.

X-ray diffraction analysis is one of the methods available to determine the crystallographic orientation of biominerals, although it reflects a statistical orientation because the X-ray beam is much broader than the domain size of an individual crystal (e.g., Checa et al., 2005). In order to determine the orientation of an individual biomineral, electron diffraction (ED) in a transmission electron microscope (TEM) is conventionally applied (e.g., Mann and Sparks, 1988). The electron back-scattered diffraction (EBSD) technique developed by Venables and Harland (1973) is commercially available and has recently been applied to biominerals (Dalbeck et al., 2006; Saruwatari et al., 2006). EBSD is also known as a backscatter Kikuchi diffraction pattern (BKDP, or BKP without “diffraction”) acquired from a bulk specimen in a scanning electron microscope (SEM) [see the review by Baba-Kishi (2002) for detailed terminology and the EBSD history].

The EBSD pattern occurs from the interaction between scattered electrons in a specimen and the crystal lattice, and consists of many band-like contrasts or Kikuchi bands, each of which corresponds to a lattice plane within the crystal. It is possible to identify the crystalline phase or polymorph and to determine the crystal
The combination of EBSD and SEM has several advantages when compared to ED in TEM, namely, easy sample preparation, a wide survey area, and superior perception of the crystal morphology or attitude. However, the minimum area the EBSD pattern can be acquired from is generally about a few hundred nanometers in width with a field-emission type electron gun (Harland et al., 1981) and is normally larger than that of ED in TEM. After the discovery of Kikuchi patterns with an overlap on the ED patterns (Kikuchi, 1928), Kikuchi pattern analysis became a common method of determining crystal orientation in TEM (e.g., Hirsch et al., 1965) because it does not require specimen tilting as compared to conventional ED analysis. However, the application of Kikuchi pattern was hitherto very limited in mineralogy and related earth science fields due to the cumbersome and time-consuming process of developing and reading film, and of calculating a crystal orientation from the pattern. Furthermore, the Kikuchi pattern obtained from a small or thin crystal like that investigated in the present study is so faint that it is difficult to record the pattern using conventional film. This problem has been overcome by the development of technology for TEM instrumentation and its associated data processing system (e.g., Zaefferer and Schwarzer, 1994). In this study, we used a recently advanced CCD camera with a large dynamic range in order to record very weak Kikuchi patterns and we demonstrate that Kikuchi pattern analysis in TEM can easily determine the orientation of small crystals within BCM magnetosome and coccolith specimens.

**APPARATUS**

A high-performance charge-coupled device (CCD) camera and a dedicated data processing program were used in order to acquire Kikuchi patterns and determine crystal orientation during TEM observation. A Gatan ES-500W CCD camera was placed just above the phosphor screen at a port of the column in a JEOL JEM-2010 (UHR) TEM operated at 200 kV in order to acquire a wide area of the diffraction pattern. The grayscale of the CCD camera is about 12 bits and extends to about 15 bits (∼35000 shades of gray) by integrating several dozen of exposures on a Gatan DigitalMicrograph (ver. 3.1 0.0). This deep grayscale is an important factor that facilitates visualization of faint Kikuchi patterns by adjusting the contrast and brightness of the original image. The maximum range of Kikuchi patterns acquired on the screen depends on the design of the TEM used. In case of JEM-2010, the maximum angle span reaches ∼13° when a small camera length, such as 200 mm in the present study, is used (Fig. 1). Patterns for higher angles appear by adjusting the current to an intermediate lens setting, but barrel-type distortion and torsion seriously affect the image quality around the outer edge. Although the maximum angle span of a Kikuchi pattern is about one-third smaller than that of a typical EBSD result in SEM, it can nevertheless determine crystal orientation uniquely.

A Kikuchi pattern is generated by the interaction between scattered electrons in the specimen and the crystal lattice, so that the convergent angle of the incident beam does not affect the pattern. On the other hand, the convergent beam can be selected to target a smaller area and obtain a more intense illumination than a near-parallel beam with a selected aperture. Such intense illumination can emphasize weak Kikuchi patterns in TEM, although it causes radiation damage to the specimen. For instance, a concentrated beam can alter calcium carbonate to amorphous phases and ultimately transform it to calcium oxide (CaO) (Walls and Tencé, 1989). In the present study, the samples were observed in the normal TEM mode (spot size: No. 2) of the JEM-2010 illumination system using a convergent beam generated with a condenser aperture of 70 μm diameter.
The Kikuchi pattern acquired by the CCD camera was analyzed similarly to the EBSD patterns using a data processing program developed by Kogure (2003). In this, three Kikuchi bands in the acquired pattern are traced to determine the crystal orientation (Fig. 1a). The calculated pattern with the same crystal orientation is drawn to examine whether other Kikuchi bands coincide between the experimental and calculated patterns to verify the results (Fig. 1b).

SAMPLE PREPARATION

The magnetosomes investigated were those of *Magnetospirillum Magnetotacticum* MS-1 (Fig. 2). The cultivation method of this strain is described in Taoka et al. (2003). The bacteria were suspended in 20 mM Tris-HCl buffer solution. After the centrifugation, the sample solution enriched in bacteria was fallen in drops onto a microgrid covered with a holey carbon film for TEM observation. The coccolith under study was a calcified scale of *Pleurochrysis carterae* (Fig. 3), which is a cricoliths type coccolith defined as an elliptical heterococcolith with elements arranged in a simple ring (Heimdal, 1993). The *P. carterae* strain was cultivated in a transparent 5 L flask containing 4 L of a seawater-based medium prepared after Eppley et al. (1967) at 19 °C with continuous aeration in a cycle of 18 hours light and 6 hours dark for 10 days. The ambient intensity of white light was about 100 μE/m²·s. The coccolith scales were isolated from the above cells according to the method reported by Marsh et al. (1992). The isolated coccoliths were washed five times with 0.05 M NaHCO₃ (pH 8.3) followed by centrifugation to remove the cellular organic materials and then rinsed once in 1.0% sodium hypochlorite to eliminate surface organic matter. After dehydration with 100% ethanol and the centrifugation, the sample solution was fallen in drops onto a microgrid for TEM observation in the same manner as the bacteria described above.

RESULTS AND DISCUSSION

**Spinel-law twin in magnetosomes**

Magnetosomes, which are intracellular magnetite (Fe₃O₄) particles surrounded by organic vesicles in magnetotactic bacteria, are fine particles several tens of nanometers in size and one of the most studied biominerals. The significance of magnetosomes in earth science has been understood from the magnetization of sediments (Stoltz et al., 1986; Akai et al., 1991) and in magnetofossils, which are related to the phylogenetic evolution of prokaryotes (Chang and Kirschvink, 1989; Akai and Iida, 1997; Akai et al., 1997). Magnetosomes came to the fore after the discovery of similar magnetite particles found in a Martian meteorite ALH84001, which might be proof of the existence of life on Mars (McKay et al., 1996). However, many abiogenic interpretations have been presented (Mikouchi et al., 1997; Bradley et al., 1998; Barber and Scott, 2002; Golden et al., 2000, 2001) and thus debate continues.

An interesting crystallographic property of magnetosomes is the occurrence of spinel-law twins with (111) as the twinned plane (Devouard et al., 1998), which is commonly observed in magnetite and other cubic miner-
Crystal orientation analyses of biminerals using Kikuchi patterns in TEM

K. Saruwatari, J. Akai, Y. Fukumori, N. Ozaki, H. Nagasawa and T. Kogure

Crystal orientation analyses of biominerals using Kikuchi patterns in TEM. To elucidate the formation mechanism of magnetosomes, it is important to know whether two or more domains in single magnetosomes may be explained by the spinel-law twin or, alternatively, as several crystals with no twin relation, because the latter case may imply multinucleation of magnetite in an organic vesicle (Gorby et al., 1988).

In general, several tens of magnetosomes form a chain in a cell (Fig. 2a). The magnetosomes in MS-1 have a cuboctahedral morphology surrounded by the {111} and {100} planes (Dunin-Borkowski et al., 2001). In our observation, some magnetosomes have different diffraction contrasts in one grain. High-resolution TEM imaging suggests the twin relation (Fig. 2b). When the magnetosomes are properly oriented in TEM, ED patterns and high-resolution TEM images can represent the twin relation. Not all twinned crystals can be oriented in TEM due to a limited tilt angle. On the other hand, Kikuchi pattern analysis is more easily performed.

The convergent beam was aimed at the points indicated in Figure 2a (1A, 1B...3B) and the acquired Kikuchi patterns are shown in Figure 4a with the calculated patterns (Fig. 4b) to match the experimental ones. Figure 4c shows stereo-nets to indicate the pole directions of the {100}, {110}, and {111} planes. As magnetite is cubic, these poles are equivalent to the zone-axis directions with the same indices. From the comparison of stereo-net pairs for the two domains with different diffraction contrasts in a single particle, it is observed that the two stereo-nets have one common {111} pole, as indicated by white arrow heads, and three common {110} poles that are normal to the {111} pole, which are shown by black arrow heads. The common {111} plane is the twin plane of the spinel-law twin and the three {110} poles normal to the {111} pole coincide by a 180° rotation between the twinned domains around the {111} pole. The orientational relations between the two domains in a single magnetosome in Figure 2 are therefore all explained by the spinel-law twin. We examined about ten magnetosomes that contained different diffraction contrasts and found no exceptions, implying that magnetite crystal multinucleation in a vesicle seldom occurs during the biomineralization of a magnetosome.

Crystal orientations of calcite in coccolith

Coccolith is an environmentally, biologically, and geologically important biomineral produced as the calcified scales of coccolithophore marine unicellular algae. In general, a single coccolith consists of several tens of calcite crystals that are interlocked together to form a small ring or disk 1-10 μm in diameter and exhibits elaborate species-specific morphology (Young and Henriksen, 2003). One of the essential approaches to understanding the biomineralization mechanism of coccoliths is a simultaneous comprehension of the crystallography and morphology of all the coccolith elements. ED in TEM has been conventionally applied to determine the crystallo-
Crystal orientation analyses of biominerals using Kikuchi patterns in TEM graphic orientation in coccoliths (Mann and Sparks 1988; Didymus et al., 1994; Marsh, 1999). Such determination using ED generally requires a pair of diffraction patterns from different directions for each crystal only when the zero order Laue zone is examined. During ED measurements, furthermore, each crystal’s tilt angle must be considered to determine the orientational relationship between adjacent crystals. As shown in the following discussion, to determine the crystal orientation in a coccolith, Kikuchi pattern analysis is more efficient than conventional ED.

The coccolith *P. carterae* consists of two kinds of interlocked units on an oval organic base plate (Marsh, 1994; Okazaki et al., 1998; Marsh, 1999). These are referred to as V- and R-units in Figure 3, and represent crystals with subvertical and subradial orientation of the c-axis, respectively (Young et al., 1997; Marsh, 1999). The image at the center in Figure 5 shows a bright-field image viewed almost perpendicular to the coccolith plane. From this direction, the V- and R-units overlap along the beam direction, rendering it difficult to take Kikuchi patterns from R-units. V-units hang over the R-units on the outer

**Figure 5.** (Upper) Kikuchi patterns from individual calcite fragments (V-unit) in the coccolith. The center image is a bright-field image of the analyzed coccolith to show the analyzed points. (Lower) Stereo-nets to show the directions of the c-axis (black arrow) and two a_1-axes (gray arrows) of calcite, determined by the analysis of the Kikuchi patterns.
side of the coccolith because of their larger size (Fig. 3). Consequently, the Kikuchi patterns could only be taken from the V-units by aiming the convergent beam at the outer side (Fig. 5). The images were clear enough to determine the crystal orientation of the calcite fragments, as shown in the upper half of Figure 5. The lower half of Figure 5 shows the projections of the e-axes and two a– axes on the stereo-nets as determined from the Kikuchi patterns. This result indicates that all the V-units are oriented in virtually the same direction relative to the circumference of the coccolith. For instance, the projections of the e-axes on the coccolith plane are all oriented about 30° inside the tangent of the coccolith ring. This result coincides with that of the recent study using EBSD analysis by Saruwatari et al. (2006).

Compared with EBSD analysis, there are advantages and disadvantages to using Kikuchi patterns in TEM.

First, Kikuchi patterns in TEM can be obtained from any part of the crystals in the view if the crystals do not overlap, whereas EBSD patterns in SEM cannot be acquired from the portions that are hidden by a specimen tilt of ~70° from the horizontal position toward the detector and by the elaborate morphology of the coccolith. The crystal orientations of all the V-units could thus be determined using Kikuchi patterns in TEM, but the orientations of the R-units were unavailable because of the overlapping with the V-units. On the other hand, both V- and R-units were analyzed using EBSD when both distal and proximal sides were observed (Saruwatari et al., 2006).

Second, the domain of a single crystal is easily identified from the diffraction contrast in the TEM image.

Third, TEM and SEM observations provide quite different morphological information. The morphology of *P. carterae* is symmetrical so it was difficult to determine the distal or proximal sides of coccoliths from transparent TEM images. Where the morphology is chiral as for other species such as *Emiliania huxleyi*, it is possible to determine the side of a coccolith from it. Because of these methodological advantages and disadvantages, Kikuchi pattern analysis in TEM and SEM (EBSD) are complementary, and both will be necessary for a complete coccolith crystallographic analysis.

**CONCLUDING REMARKS**

Although Kikuchi pattern analysis in TEM is an orthodox technique, it is still very useful in determining the crystal orientation in specimens, especially with a regulated multocrystal assembly. As demonstrated in this study, in a manner similar to EBSD, TEM analysis can be rapid and easy with the recent developments in recording media and software. Furthermore, the magnetosome study has also demonstrated the high spatial resolution of this technique. Other than its pertinence to the study of biominerals, there must be many other possible applications of this technique in mineralogy and related earth science fields.

**ACKNOWLEDGMENTS**

We thank Dr. S. Uehara and Dr. C. Numako for their reviews and Dr. M. Kawano for handling the manuscript. Dr. M. Okazaki of Tokyo Gakugei University kindly provided coccolith material. This work was supported by a Grant-in-Aid for scientific research (No. 17GS0311) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**REFERENCES**


Royal Society of London Series B-Biological sciences. 258, 237-245.


Venables, J.A. and Harland, C.J. (1973) Electron backsceattering patterns – New technique for obtaining crystallographic in-


Zaefferer, S. and Schwarzer, R.A. (1994) Automated measure-
ments of single grain orientations in the TEM. Zeitschrift für Metallkunde, 85, 585-591.

Manuscript received June 11, 2007

Manuscript accepted September 14, 2007

Manuscript handled by Motoharu Kawano