Crystallographic relationships in the crossed lamellar microstructure of the shell of the gastropod *Conus marmoreus*

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ABSTRACT

The crossed lamellar microstructure of mollusk shells shows a very complex hierarchical architecture constituted of long rod-shaped aragonite crystals stacked parallel to each other inside each first order lamella, which are almost perpendicular to the ones contained in parallel neighboring lamellae. To better understand the construction and properties of the crossed lamellar microstructure we have performed a detailed study to determine the crystallographic characteristics and their evolution during shell growth using scanning electron microscopy, transmission electron microscopy and X-ray diffraction texture analysis. The arrangement of crystals is rationalized by a set of twin law relationships between aragonite crystals. Specifically, the aragonite rods, or third order lamellae within each first order lamella, internally consist of polysynthetic twins bounded by {110} mirror planes. In turn, the polysynthetically twinned aragonite crystals also show a constant crystallographic orientation with respect to aragonite crystals in adjacent first order lamellae. It can be seen as another twin law in which crystals from adjacent lamellae are bounded by (110) planes but with their *c*-axes rotated within this plane by 30°. Thus there are two sets of twin laws that relate crystal units at lower (third order lamellae) and higher (first order lamellae) length scales. These hierarchical relationships play a crucial role in the construction, organization and properties of this complex microstructure. The later orientational relationships have never been described in geological aragonite and are only found in biogenic materials with a crossed lamellar microstructure. Their occurrence is probably determined by the presence of shell organic components which regulate crystal growth and may favor unusual crystallographic relationships.

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1. Introduction

During their evolution mollusks and other organisms have developed exceptionally strong protective shells which are built from minerals and organic materials (i.e. calcite, aragonite, apatite, etc.) that are intrinsically fragile [1,2]. One strategy is to reinforce the crystals with macromolecules that are incorporated into the crystal structure, thus preventing easy cleavage. In addition to macromolecules used to glue crystals together, another complementary strategy is to organize the constituent crystals into very complex microstructural architectures. Each microstructural organization confers distinctive mechanical properties on the shells. In particular, shell microstructures such as nacre and crossed lamellar (CL) layers, which have a nanoscale laminate architecture, are the toughest. The CL microstructure has a very complex architectural organization of aragonite crystals (see Fig. 1). It consists of parallel lamellae (first order lamellae), which are in turn composed of long aragonite rods (third order lamellae). Those aragonite rods run parallel to each other within first order lamella but form a high angle with those constituting adjacent first order lamellae (Fig. 1A–E). First order lamellae are in turn packed into second order lamellae through the lateral connection of adjacent rods along the direction perpendicular to the plane containing the first order lamellae (Fig. 1E and F). They consist of planar arrangements of laterally adjacent third order lamellae, the planes being perpendicular to those containing the first order lamellae (see Fig. 1F and the sketch in Fig. 5). The resulting laminated ceramic composite has a complex architecture reminiscent of that of plywood [3,4]. The distribution of this microstructure in molluscan species has increased
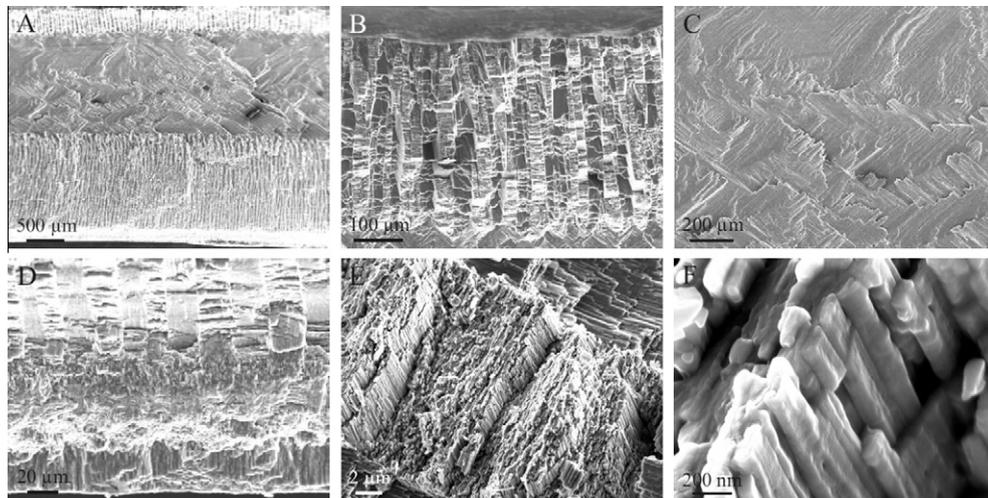
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Fig. 1. (A–D) SEM micrographs of the microstructural organization of *Conus marmoreus* shell. (A) Co-marginal fracture showing the outer (top), middle and inner (bottom) crossed lamellar layers. (B) Detail of the outer crossed lamellar layer, with first order lamellae perpendicular to the fracture plane and parallel to the shell growth (longitudinal) direction. (C) Middle layer, showing lamellae parallel to the fracture (co-marginal) plane. (D) Detail of the internal crossed lamellar layer (with first order lamellae oriented longitudinally) and of the underlying fibrous layer (bottom of photograph). (E, F) Details of the crossed lamellar shell of *Conus omaria*. (E) Fracture showing the varied inclinations of the second order lamellae in alternating first order lamellae. (F) Detail of (E) showing the plate-like elongated aspect of the third order lamellae and their lateral arrangement into second order lamellae.

over geological time until it has become the dominant microstructure in most molluscan classes (except for the relict monoplacophorans). The reason for its evolutionary success may be explained by its superior mechanical strength, which is due to the arrangement of aragonite crystals as a laminated microarchitecture that restricts microcrack propagation and makes this material exceptionally tough against fracture [4]. This complex architecture confers on the CL microstructure a mechanical advantage that exceeds even that of nacre (another laminated shell microstructure considered to be the toughest among mollusk shell microstructures). In particular, the work to fracture of the CL microstructure has been reported to be 10 times higher than that of nacre [5]. Based on the exceptional performance of these biomaterials, some advanced ceramic materials which reproduce specific shell microstructures are currently being designed [6]. Additionally, the CL microstructure is probably metabolically cheap to produce (as it contains 1% organic matter, compared with 4.5% in nacre) [7]. According to Uozumi et al. [8] the organic matter is mainly distributed as thin organic sheaths enveloping the third order lamellae.

The CL microstructure has been studied by several authors [3–5,8–12] using different analytical techniques (X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM)). For detailed descriptions of the morphology and mode of growth of the CL layer see Carter [3]. Nevertheless, only a few authors [9,10,12] have described the crystallographic orientation of crystals forming this complex architecture, which certainly deserves revision using the new techniques that are now available to define crystallographic relationships (i.e. preferential, orientation, epitaxy and twin laws) that may exist in the materials. Moreover, there are no studies describing the evolution of the crystallographic properties with shell growth. Thus we have undertaken a detailed study of the crystallographic characteristics of this type of material to precisely determine the crystallographic relationships among crystals on different length scales and their evolution through the different CL shell layers of the neogastropod *Conus marmoreus*. High resolution field emission (FES-EM), XRD texture and TEM analyses have been used to determine the crystallographic arrangement and to observe whether it is determined by the crystal structure (epitaxy and twin laws). FESEM can provide highly detailed information on the size, morphology and disposition of crystal units making up the shell. However, it provides only limited information on the crystal orientation, since the morphology alone is an insufficient criterion for the identification of the main crystallographic directions. This is particularly true in the case of the CL microstructure. XRD texture analysis is more suitable and allows a precise determination of the statistical distribution of crystal orientations within the mineral assemblage. This technique provides detailed information on the disposition of crystal orientations in three dimensions at the millimeter scale. At much higher spatial resolution, TEM analyses provide highly detailed information about the morphology and crystallographic orientation of constituting units at the nanoscale. A combination of these complementary techniques can lead to a more complete picture of the complex organization and structure of the CL microstructure at different levels. Knowledge of the interrelations of structural units is essential to better understand the mechanisms controlling the formation of these materials and their functional properties and, finally, a fundamental basis to the quest to reproduce them in the laboratory.

2. Materials and methods

2.1. Materials

Several well preserved specimens of the Indo-Pacific neogastropod species *Conus marmoreous* Linnaeus, 1858, *Conus striatus* Linnaeus, 1858 and *Conus omaria* Hwass in Bruguière, 1792 were selected. All specimens were purchased from Conchology Inc.

2.2. Scanning electron microscopy

SEM observation of the CL microstructure was carried out on both fractured and polished sections of the shell. Samples were usually observed intact, although in some we removed the organic matter (with 5% NaOH for 1–2 h or proteinase K (1 mg ml^{-1}) for 1–20 min) or, in the case of polished sections, the surface was slightly etched using diluted acid (1% HCl) at room temperature. Samples were carbon coated (Hitachi UHS evaporator) for FESEM (Leo Gemini 1530) or environmental SEM (ESEM) (FEI Quanta 400) observation.

2.3. X-ray diffraction

To perform a depth profile analysis by XRD a 1 cm wide strip of *C. marmoreus* shell was cut parallel to the shell edge (i.e. parallel to the shell elongation axis) (see Fig. 2). Subsequently this strip was embedded in resin and cut and polished in a slightly tilted way with respect to the outer shell surface to produce a wedge in which all the shell layers were exposed across it. In one extreme of the strip the corresponding shell thickness was 0 (outer surface) and on the other extreme it was 100% of the total shell thickness (inner surface). XRD analyses were performed along the strip every 2 mm (the X-ray beam was 1 mm in diameter). Once the XRD analyses had been performed the shell strip was finely polished and etched with 2% EDTA. It was subsequently observed by ESEM (FEI Quanta 400) to obtain a detailed record of the orientation of the first order lamellae all along the strip length.

The X-ray experiments were carried out using a D8 Bruker diffractometer equipped with a Euler cradle, parallel beam optics of PolyCap type and a special sample holder attached to the x–y–z sample stage. The change in orientation of crystals across the shell thickness was assessed by three-dimensional (3-D) texture analysis. Incomplete back-reflection pole figures $(1\,1\,1)$, $(0\,1\,2)$ and $(0\,0\,2)$ were registered at each measurement position. The LaboTex package (Labosoft) was used to calculate the ODF function and the complete pole figures at each measuring point, using the discrete ADC method [13]. For these analyses we considered that the material is pure aragonite (symmetry: $2/m2/m2/m$; $a_0 = 4.9623$ Å, $b_0 = 7.9680$ Å, $c_0 = 5.7439$ Å). For this material the effective penetration depth of CuK_α radiation is 70 µm for the $(1\,1\,1)$ diffraction reflection, while the estimated difference in shell thickness between two consecutive measuring positions is 140 µm.

2.4. TEM analyses

For TEM analyses specimens of *C. marmoreus* were first mechanically polished and subsequently thinned down to electron transparency with a GATAN precision ion polishing system (PIPS) at the 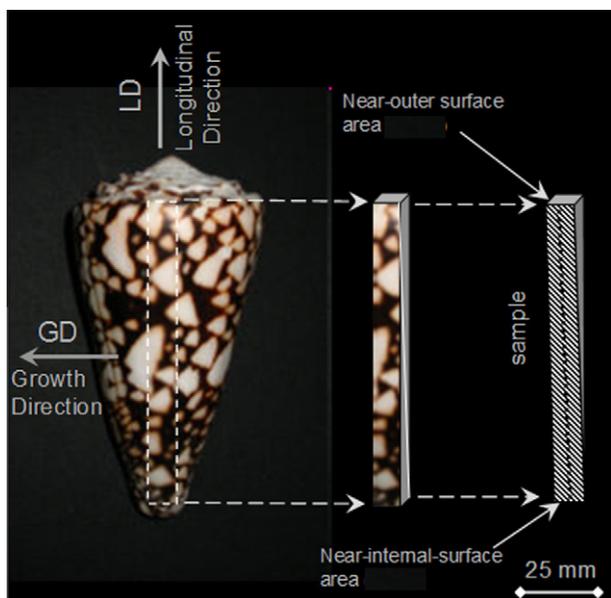Max-Planck Fritz-Haber Institute in Berlin, Germany. TEM analysis was carried out using a Jeol 2200FS TEM microscope at the University of Aveiro, Portugal.

3. Results and discussion

3.1. Scanning electron microscopy

The shells of the studied species of *Conus*, when fully grown, are composed of three superimposed CL layers: an outer layer in which the first order lamellae are longitudinally arranged (i.e. parallel to the main elongation axis of the shell), an intermediate layer with first order lamellae arranged radially, and an internal layer with first order lamellae arranged longitudinally. Thus the three CL layers are structurally identical except that the plane containing the first order lamellae is rotated by 90° with respect to that of the previous layer (Fig. 1A and D). In all cases the planes containing the first order lamellae are always perpendicular to the shell surface (Fig. 1A). Additionally, there is a thin innermost layer made of fibrous aragonite. In the *Conus omaria* shell the layer thicknesses were 350–424 µm (external CL layer), 1380–1600 µm (middle CL layer), 1580–1650 µm (internal CL layer) and 95–100 µm (innermost fibrous layer) (see Fig. 1A–D).

First order lamellae have a variable thickness of between 10 and 30 µm, which varies with depth. Some lamellae even thin and disappear, while others initiate and thicken (Fig. 1A, B and D). Third order lamellae are long rod-shaped aragonite units with a rectangular cross-section, about 100 nm thick and 150–350 nm wide (Fig. 1F). Third order lamellae units from adjacent lamellae dip in opposite directions and form an angle of about 105° (Fig. 1C–E). The third order lamellae are bundled into unique thick layers, which are arranged perpendicularly to the planes between the first order lamellae; they form the so-called second order lamellae. The transition between the distinct superimposed CL layers making up the shell is relatively abrupt and takes place across a horizontal transition zone of some 50 µm (Fig. 1B).

3.2. Transmission electron microscopy

As previously described, the basic building units of the CL microstructure are long aragonite crystal rods with a rectangular cross-section, known as third order lamellae. Fig. 3A shows a TEM image of a cut perpendicular to the longest axis of bundled third order lamellae. Each third order lamella displays an internal structure consisting of straight nanometric bands with parallel boundaries. These banding structures are indicative of the existence of polysynthetic twins of aragonite. The bands are approximately parallel to the side faces bounding adjacent third order
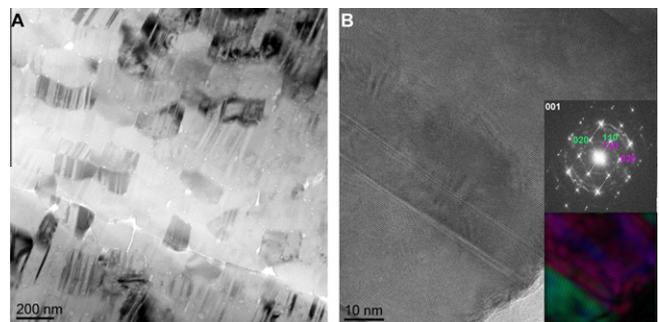

Fig. 2. Scheme showing the geometry of the shell strip sample used for XRD analyses. The 1 cm wide strip was cut parallel to the elongation axis of the *Conus marmoreus* shell and obliquely sectioned to expose the different shell layers along it.

Fig. 3. (A) TEM image viewed perpendicular to the elongation axis of third order lamellae which are internally constituted by polysynthetic twins. (B) High resolution image showing two adjacent twin domains within a third order lamella. The lower inset is a false color image which shows that the twinned domains have different crystallographic orientations. The FFT pattern of the lower inset image shows that the two twin domains are related by a $(1\,1\,0)$ twin mirror plane.

lamellae. However, in Fig. 3A it can be seen that there is some degree of misorientation between bands in adjacent third order lamellae (up to 30°).

Fig. 3B displays a high resolution TEM image showing adjacent twin domains within a third order lamella. In the lower inset a false color image obtained by Fourier filtering using spots associated with the different twin domains shows that they have different crystallographic orientations. The orientation relationship between domains is indicated in the fast Fourier transform (FFT) of the high resolution image shown as an inset. It is evident that the FFT pattern has a pseudo-hexagonal symmetry arising from the combined diffraction of two domains bounded by a {110} mirror twin plane. Interestingly, it has been stated that aragonite twinning in CL structures always occurs on the same (110) plane [9,12], unlike other shell microstructures, such as mollusk nacre [14,15], and geological aragonite, in which twinning also occurs in the symmetrically equivalent (1$\bar{1}$0) plane [16]. In any case, the occurrence of twinning in only one plane partly explains the occurrence of polysynthetic twins instead of cyclic twins, in which twining occurs in both the (110) and (1$\bar{1}$0) planes of aragonite. Cyclic twins are the most commonly found twin law in aragonite and is favored by the pseudo-hexagonal symmetry of this mineral structure [16]. On the other hand, it has been shown that fibrous aragonite crystals in other shell microstructures also have a high density of (110) twins [15]. The formation of these growth twins is probably favored by the high rate of aragonite deposition in these specimens [12,15,16].

3.3. X-ray diffraction

The (001) pole figures obtained at different shell thicknesses show two well-defined maxima except at the outer surface, where there is a diffuse and very broad central maximum (Figs. 4A (top) and 5). These two maxima are displayed near the center of the pole figure and are separated by an angular distance of about 28–31°. On the other hand, the (100) pole figures show four distinct maxima near the rim of the pole figure (Fig. 4A (bottom)). The disposition of maxima in pole figures imply that there are two sets of crystals. The position and distribution of the two maxima displayed on the left side of the (100) pole figure are associated with the 001 maxima on the right and correspond to one set formed by two crystal units that have their c-axes aligned but have their a-axes rotated 60° around this direction. The later crystallographic relationship is equivalent to that described for polysynthetic twins constituting third order lamellae (observed by TEM), that are related by a (110) twin plane (see Fig. 3). The other set of two crystals would produce the 001 maxima on the left and two 100 maxima on the right. Again, the second set is related by a (110) twin mirror plane. On the other hand, the two sets of crystals have their crystal c-axes contained in the plane of the first order lamellae but are tilted 14–15°, in opposite directions, with respect to the shell surface normal (see Fig. 4B).

Interestingly, the two sets of (110) twinned crystal units are in turn related by a well-defined recurrent relationship. The crystallographic relationship between the two sets of crystals can be
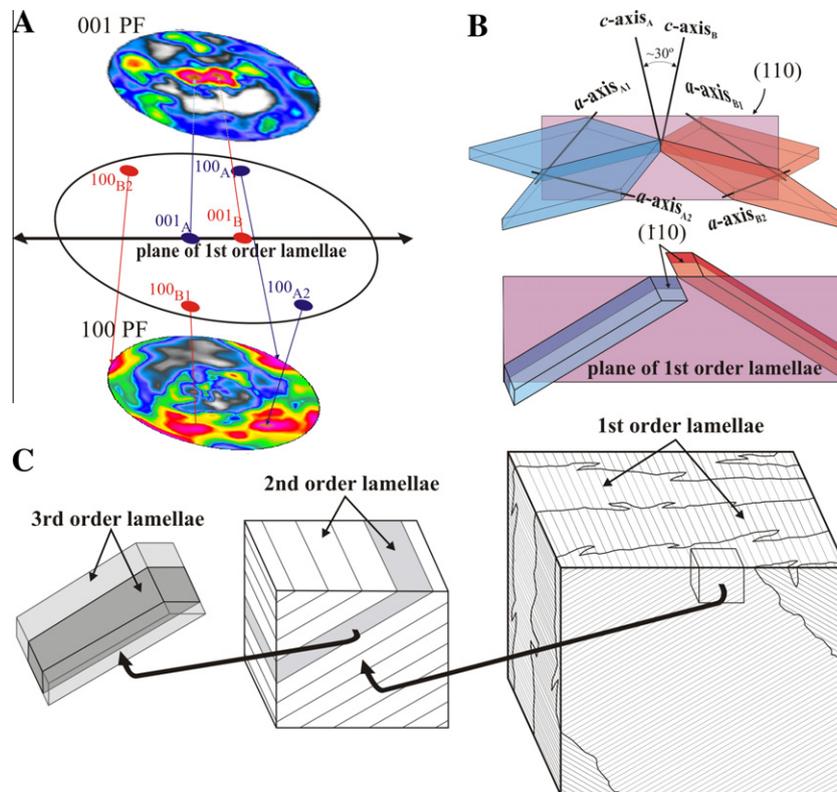

Fig. 4. Model for the orientation distribution and twinning relationships between crystals in the crossed lamellar microstructure. (A) Sketch of the (001) and (100) pole distributions. The pattern implies that there are two sets of crystals (A and B) with their c-axes contained within the plane of the first order lamellae but tilted 30° relative to each other. Each 001 maximum has two associated 100 maxima rotated by 60°, implying that there are two crystals related by a mirror (110) twin plane. This orientation relationship is the same as that relating polysynthetic twin domains composing third order lamellae (Fig. 3A). The two sets of twinned crystals (with different 001 maxima) belong to adjacent first order lamellae and are in turn related by a composition (110) plane and rotated about the normal of this plane by 30°. (B) (Top) 3-D reconstruction of the two set of crystals arranged so that they reproduce the pole figure distribution in (A). (Bottom) 3-D morphological model showing that the two sets of crystals belong to two adjacent first order lamellae. (C) Sketch showing the crossed lamellar microstructure and its constituent first, second and third order lamellae units. First order lamellae are thick layers oriented perpendicular to the shell outer surface and are internally constituted of aragonite rods (third order lamellae). The rods of adjacent lamellae form a high angle. The third order lamellae are in turn disposed in laminar packs (second order lamellae).
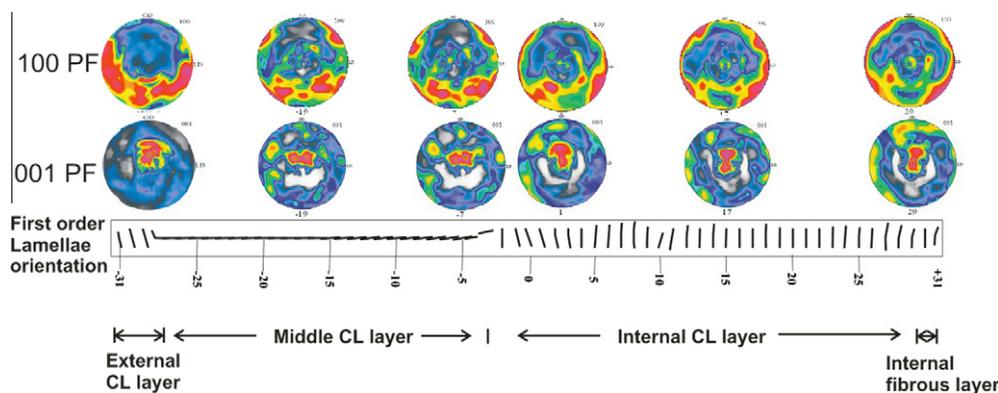
Fig. 5. Evolution of crystal orientation across the shell thickness and its relationship to first order lamellae. (Top) XRD (0 0 1) and (1 0 0) pole figures obtained at six selected positions (out of the 63 positions analyzed along the shell strip). The lower sketch shows the disposition of first order lamellae planes as solid lines over the strip. The distribution and extension of the CL shell layers are also indicated below the strip. The two and four well-defined maxima displayed in the 0 0 1 and 1 0 0 pole figures are indicative of the occurrence of two sets of intertwinned crystals. Note the progressive 90° rotation of first order lamellae at the transition between different CL shell layers. In all cases the two maxima in the 0 0 1 pole figures are always parallel to the orientation of the first order lamellae. Rotation does not occur between the internal CL layer and the innermost fibrous layer.

described as a new twin law in which the two sets of related crystals are bounded by parallel (1 1 0) planes but the crystals are rotated around the normal to this plane by 30° (so that their associated *c*-axes are rotated by 30°) (see Fig. 4B). This crystallographic relationship has not been observed by TEM between crystal domains making third order lamellae, which are exclusively related by (1 1 0) twin mirror planes. On the contrary, this new orientation relationship must relate third order lamellae belonging to adjacent first order lamellae which have their *c*-axes rotated 30° relative to each other but are bounded by parallel (1 1 0) planes, as shown in the lower sketch in Fig. 4B. This orientation relationship appears not only in the CL microstructure of *Conus* but also in other mollusk species with this type of microstructure [10] (personal observation). The shell organic matter must favor the development of such uncommon crystallographic relationships [17,18]. A similar orientation relationship was described by Wilmot et al. [9]. The latter authors suggested that this orientation relationship between third order lamellae is caused by some form of biological switching of the orientation of the growth planes. However, the constant misorientation between the two sets of *c*-axes (30 ± 2°) is more indicative of a crystallographically controlled relationship (a twin law in this case).

Fig. 5 shows how first order lamellae (displayed as solid lines over the strip) are rotated by 90° at specific positions along the strip that correspond to the transition zones between adjacent CL layers. However, the rotation of first order lamellae is not abrupt but progressive within the transition zone. The relative position and distribution of the maxima in the pole figures is nearly constant at all measured positions except that the maxima are rotated by 90° in different CL layers so that two maxima in (0 0 1) pole figures are consistently aligned parallel to the orientation of the first order lamellae. Thus the orientation of the crystals is rotated coincident with first order lamellae so that their *c*-axes are always contained in the plane of the first order lamella, while the *a*-axes are out of this plane by about 60°. On the other hand, SEM observation reveals that there is always continuity of growth of the crystals in these transition zones between CL layers. One possibility is that rotation of the lamellae in the transition zone between adjacent CL layers could also be determined or facilitated by some form of crystal twinning. However, this type of twinning was not evident in this study. It is also interesting that the crystallographic orientation of the crystals is preserved at the transition between the inner CL layer and the innermost fibrous layer. This is most probably due to epitaxial nucleation of fibers on the previous CL layer.

4. Conclusions

We have studied the changes in crystallographic orientation through the different CL shell layers in the gastropod *C. marmoreus*. Within each CL layer the crystals are related by twin laws in which there are two sets of crystal units. Within each set there are crystal twin domains related by a (1 1 0) mirror twin plane. This type of relationship occurs within third order lamellae which are constituted of polysynthetically twinned aragonite. On the other hand, the two sets of crystals share a common (1 1 0) plane, but are related by another twin relationship by which their *c*-axes are rotated around the normal of this plane by 30°. This relationship appears between crystal units belonging to adjacent first order lamellae. The shell organic matter must favor the development of such uncommon crystallographic relationships. In conclusion, twin laws between structural units play an important role in the construction and organization of such an intricate microstructure.

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figures 2–5, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.actbio.2011.11.001.

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