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Crystallographic control on the substructure of nacre tablets

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ABSTRACT

Nacre tablets of mollusks develop two kinds of features when either the calcium carbonate or the organic portions are removed: (1) parallel lineations (vermiculations) formed by elongated carbonate rods, and (2) hourglass patterns, which appear in high relief when etched or in low relief if bleached. In untreated tablets, SEM and AFM data show that vermiculations correspond to aligned and fused aragonite nanogloblules, which are partly surrounded by thin organic pellicles. EBSD mapping of the surfaces of tablets indicates that the vermiculations are invariably parallel to the crystallographic *a*-axis of aragonite and that the triangles are aligned with the *b*-axis and correspond to the advance of the {010} faces during the growth of the tablet. According to our interpretation, the vermiculations appear because organic molecules during growth are expelled from the *a*-axis, where the $Ca–CO_3$ bonds are the shortest. In this way, the subunits forming nacre merge uninterruptedly, forming chains parallel to the *a*-axis, whereas the organic molecules are expelled to the sides of these chains. Hourglass patterns would be produced by preferential adsorption of organic molecules along the {010}, as compared to the {100} faces. A model is presented for the nanostructure of nacre tablets. SEM and EBSD data also show the existence within the tablets of nanocrystalline units, which are twinned on {110} with the rest of the tablet. Our study shows that the growth dynamics of nacre tablets (and bioaragonite in general) results from the interaction at two different and mutually related levels: tablets and nanogranules.

1. Introduction

The first crystallographic data on the nacre tablets were reported by Schmidt (1922), who concluded with optical microscopy that the *c*-axes of aragonite were perpendicular to tablets. In his subsequent papers, Schmidt (1923, 1924) used the outlines of tablets to infer the positions of {110}, {100} and {010} faces and, hence, the orientations of the *a*- and *b*-axes of the tablets. With the advent of the scanning electron microscopy, later authors e.g., (Grégoire, 1962; Wada, 1972; Mutvei, 1969, 1970, 1972a,b) refined that knowledge, but the basic schemes provided by Schmidt remained essentially unaltered.

X-ray diffraction techniques are useful for determining crystal orientation, but its application is hindered by the fact that they can only determine the orientation of groups of tablets in relatively wide areas (at least 250 μm in diameter). An additional handicap is that, due to the deep penetration of X-rays, the results combine data from dozens of superposed lamellae. Nevertheless, some insight can be gained. The distributions of maxima of pole figures indicate that in nacre tablets the *c*-axes are perpendicular to the main surfaces. Polarization-dependent imaging contrast (PIC), which is based on X-ray linear dichroism, has also been extensively applied to bivalve and gastropod nacres (Metzler et al., 2008; see review in Gilbert (2012)). It is a high resolution method (~20 nm), but can only detect changes in the *c*-axis, and not in the *a*- or *b*-axis. Additionally, the tablets of bivalves are known to be co-oriented, with the *b*-axis pointing in the local growth direction of the shell (Wada, 1961, 1972; Wise, 1970; Checa and Rodríguez Navarro, 2005; Rousseau et al., 2005). Tablet faces can sometimes be indexed by checking the shapes of the tablets against the overall orientation deduced from X-ray pole figures. Transmission electron microscopy (TEM) as well as electron

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back-scattered diffraction (EBSD) coupled to scanning electron microscopy (SEM) offer much higher resolution, but application is limited because the material has to be either sliced and thinned to tens of nm, in order to make the sample transparent to ions for TEM, or sectioned and finely polished, to eliminate differences in relief, to which the EBSD technique is very sensitive. Since, the 3D aspect is lost in this way, it is difficult to relate the shape of the tablets to the orientations found. Therefore, electron diffraction techniques have been routinely used to determine crystallographic orientations only of sectioned nacre tablets (e.g., Gries et al., 2009; Dalbeck et al., 2006), but not in plain view.

In view of the above explanation, the main information currently available on the crystallography of individual nacre tablets is not always reliable, particularly taking into account their high variety of shapes (Wise, 1970; Wada, 1972; Checa and Rodríguez-Navarro, 2005).

In a number of informative papers, (Mutvei, 1970, 1977, 1978, 1979, 1980, 1991; Mutvei and Dunca, 2008, 2010), using special etching protocols, revealed the existence of two kinds of etching features. First, in the tablets of bivalves, gastropods and *Nautilus*, a series of parallel lineations that appear, are inferred to be parallel to the *a*-axis of aragonite. The same patterns have been found in other examples of biogenic aragonite which were naturally or artificially etched: foliated and prismatic aragonite (Checa et al., 2009). Second, in some bivalves (*Mytilus*, *Nucula*, and *Unio*), Mutvei (1977) found two triangular sectors on each tablet joined by a vertex at the center of the tablet (hourglass-like), which were more resistant to etching than the rest of the tablet. According to the interpretation of Mutvei (1977, 1978, 1979, 1980, 1984), this pattern arose because each pseudohexagonal tablet was in fact composed by four twinned crystals, although this model (Mutvei, 1977, Fig. 1) was found to be incorrect by Akai and Kobayashi (1993).

The patterns revealed by Mutvei clearly have some bearing on the ultrastructure of nacre tablets and therefore are worth investigating. First, we needed to compile reliable data on the crystallography of individual nacre tablets in order to unequivocally relate the etching lineations and triangles to particular crystallographic directions. This was achieved by high-resolution diffraction techniques, particularly SEM-EBSD on the surfaces of the tablets. Additional information was gained from AFM and FESEM techniques. A model for the formation of the observed features was finally proposed.

2. Materials and methods

2.1. Treatments

Etching. Specimens were etched as originally described by Mutvei (1977, 1978, 1979), the protocol consisting of (1) cleaning the tablets with a sodium hydroxide, followed by (2) etching the tablets and fixing the organic matrix at the same time with a mixture of glutaraldehyde and acetic acid. Specimens were treated at the University of Granada with the following protocol: Sodium hypochlorite 10% (step 1) for 2–30 min → glutaraldehyde 25% + acetic acid 1% in cacodylate buffer (step 2) for 2–10 min → sodium hypochlorite 1% (step 3) for 1–5 days (optional). In all cases, samples were washed repeatedly and oven dried. Bivalves:*Acila divaricata* (Turtle Island, Taiwan), *Perna viridis* (loc. unknown, Indonesia), *Pinctada margaritifera* (French Polynesia), *Anodonta cygnea* (Mira, Portugal); Gastropods: *Bolma rugosa* (Granada coast, Spain), *Gibbula cineraria* (Quiberon, France), *Gibbula umbilicalis* (São Jacinto, Portugal); cephalopod: *Nautilus pompilus* (loc. unknown).

The shells had been preserved dry and clean and we studied nacre areas which were not marginal. Etching intensity was proportional to treatment time (compare Fig. 1A–E), but it also varied between species undergoing the same treatment. No particular combination of times was found ideal for all species.

Additional observations were made at the Swedish Museum of Natural History on the nacre of the bivalve *Mytilus edulis* (coast of Sweden) and on the septal nacre of *Nautilus pompilius* (Salomon Islands). These were treated with sodium hypochlorite (25 wt.%) followed by immersion in Mutvei's solution (1:1 mixture of glutaraldehyde and 1% acetic acid to which alcian blue is added; see Schöne et al. (2005)) under constant stirring at 35–40 °C and then dehydrated in graded alcohol concentrations.

In all cases, the exact treatment times are provided in the corresponding figure captions.

Protease. Samples of the bivalves *A. divaricata* (Turtle Island, Taiwan), *Pinna nobilis* (coast of Almería, Spain) and *Pteria hirundo* (Fuengirola, Spain) were incubated in a solution of proteinase-K (US Biological) 0.1 or 0.2 g/ml for one to 2 h at 30 °C. After deproteinization, samples were washed three times for ten min under constant stirring in saline solution (PBS) and additionally washed for ten min in milli-Q water. The samples were oven dried at 45 °C and stored for later SEM examination.

Untreated and bleached samples. The nacres of the above-mentioned species, as well as those of the bivalves *Atrina pectinata* (Fuengirola, Spain), *Anodonta anatina* (River Thames, UK), *Potomida littoralis* (loc. unknown, Spain), *Isognomon radiatus* (Olango Island, Philippines) and *Neotrigonia margaritacea* (Anxious Bay, Australia) were also investigated intact, although in some cases the tablets were cleaned of organic matter with commercial bleach (4% active Cl) from 2 to 10 min.

2.2. Scanning electron microscopy (SEM)

Samples were coated with carbon (Hitachi UHS evaporator) for FESEM observation (Zeiss Leo Gemini 1530 and Zeiss Auriga CrossBeam Station) at the Centro de Instrumentación Científica (CIC) of the Universidad de Granada (Spain). Specimens in Figs. 1 and 2B and E were gold-coated and photographed with a SEM Hitachi S-4300, at the Natural History Museum, Stockholm (Sweden).

2.3. Electron back scattered diffraction (EBSD)

To relate the crystallographic data with the features detected on the surfaces of the tablets, samples were analyzed unpolished with the surfaces of the tablets placed as parallel as possible to the detector screen; only the organic matter was removed with 5% NaOCl from the samples prior to analysis. Since this technique is very sensitive to surface irregularities, the percentage of indexable patterns dropped drastically compared to polished samples, although the number of available data provided relevant information. We used two sets of equipment. First, we used an Inca Crystal (Oxford Instruments) detector coupled to a Gemini-1530 (Carl Zeiss) FESEM (CIC, Universidad de Granada). To avoid excessive charging, samples were coated with a thickness of 2 nm of carbon in a Baltec MED 020 electron beam evaporator. Samples of the nacre from the gastropod *G. cineraria* and from the bivalves *A. divaricata* and*P. margaritifera* were examined in this way. Also, samples of the nacre of the bivalves *Neotrigonia gemma* (off Cronulla, Australia) and *A. divaricata* were analyzed after sectioning and polishing. The second set of equipment was a TSL OIM detector coupled to FEI Field Emission Gun (FEG) SEM Quanta 3D microscope of the Institute of Metallurgy and Materials Science of the Polish Academy of Sciences (IMIM, Krakow, Poland). Operation in low vacuum mode made coating unnecessary. A special cone was attached to the SEM pole piece to minimize the so-called "skirt effect" of the primary electron beam and reduce the gas-path length. Analysis software (TSL OIM version *5.3*) was used to post-process the*EBSD* measurements. All data with a confidence index (CI)

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