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# Controlled Occlusion of Proteins: A Tool for Modulating the Properties of Skeletal Elements

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# CONTROLLED OCCLUSION OF PROTEINS: A TOOL FOR MODULATING THE PROPERTIES OF SKELETAL ELEMENTS

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*Dedicated to the Memory of Margaret Etter*

Abstract Composite materials in which the organic host is stiffened by guest particles, are widely used in nature and are produced synthetically by man. Organisms also produce a different type of composite in which the host is a crystal and the guests are macromolecules occluded in an orderly fashion within the crystal. The best studied examples, to date, are biogenic calcite crystals, and in particular those formed by the echinoderms. *In vitro* experiments with calcite crystals grown in the presence of echinoderm intracrystalline proteins, show that these macromolecules are occluded inside the crystal on specific planes, and their presence alters the mechanical properties of the crystal host. Furthermore, the proteins also influence the crystal textural properties. Model studies using crystals of dicarboxylic acid salts grown in the presence of intracrystalline proteins show that the coherence length is reduced in directions perpendicular to the planes on which the proteins adsorb. We found anisotropic effects in almost all the biogenic calcite crystals we examined. Furthermore, we noted an interesting relationship between the variations in coherence length in the different crystallographic directions and the gross morphology of the single crystal elements, suggesting that these proteins may also function in determining the morphology of the crystal during growth. These novel single crystal-protein composites may be just one example of strategies used in nature for producing materials with special properties.

## INTRODUCTION

Composite materials are widely utilized by man. Long before the term was coined or the concept understood, composite materials existed in nature, and were exploited by organisms. Their superior mechanical properties are

usually derived from the combination of a stiffening component embedded in a pliant organic matrix. Organisms have been using such materials for over 550 million years, when the first skeletal tissues evolved. Common examples are shells, composed of calcium carbonate crystals grown inside a predeposited protein and polysaccharide matrix, and bones, where crystals of the calcium phosphate mineral dahllite (carbonated apatite) are formed inside collagen fibers.[1]

Recently we have discovered that widely divergent organisms belonging to various phyla produce a different type of composite material barely, if at all, used by man.[2,3] The basic concept of combining a stiff component with a pliant one still applies, but the roles are reversed. In these peculiar composites, the host is a single crystal and the guests are macromolecules, deliberately occluded into the single crystal along specific crystal planes. The presence of the occluded polymers does not destroy the integrity of the crystal, but can change the material properties of the mineral. In this way, nature has overcome, at least in part, the problem of using commonplace materials that do not possess optimal mechanical properties, for constructing mineralized hard parts. The cases that we have investigated all involve calcite as a crystalline matrix, although we suspect that the same strategy may be used for different crystals.

Calcite, when used for material construction, has one major weakness, its brittleness. This is due to the existence, within the single crystal, of structurally defined planes of favored cleavage, that cause the crystal to fail in a brittle fashion well below its theoretical strength. The process of formation and propagation of cracks requires energy that is involved in the creation of new surfaces (strain energy). In crystals, the strain energy is concentrated along defined planes that act as stress concentrators, where a crack propagates smoothly, until fracture results. We proposed that the macromolecules, introduced along planes that are oblique to the cleavage planes of calcite, strengthen the material against failure by both absorbing and deviating the advancing cracks. One or more of the mechanisms well known in materials science may be involved, namely plastic yield of the material remote from the crack tip, plastic deformation at the crack tip, increase in area of the fracture surface and dissipation of energy by deformation of the polymer itself.[4]

Here we shall review some of the data relevant to this hypothesis, especially in relation to measurements of crystal texture. Measurements

were performed on some synthetic crystals with and without occluded protein, in order to develop the basic tools and establish their reliability. Most of the information, however, concerns a series of biogenic single crystal skeletal elements that are formed by different organisms, following the strategy discussed above.



## SINGLE CRYSTAL SKELETAL ELEMENTS FROM ECHINODERMS. A TEST CASE

Already at the beginning of the century, Merker discovered that the calcite crystals deposited by echinoderms are not identical to their synthetic or geological counterparts.[5] For example, their indices of refraction of light are different. He suspected that the presence of occluded organic material might be responsible for these small, but substantial differences. Since then, the presence of organic material inside biogenic crystals was repeatedly confirmed, giving rise to a never ending debate as to whether it is incidentally or deliberately occluded, whether it has a functional purpose, or even whether the host crystal could be regarded as a single crystal.[6-9]

The best known and possibly the most striking examples of skeletal elements composed of single crystals are found among the echinoderms, a class of organisms including sea urchins, brittle stars and sea stars. It has long been known, initially from polarized light experiments and subsequently from X-ray diffraction studies, that the tests, plates, ossicles and spines of these animals are built of discrete, sometimes huge, single crystals of calcite.[7] Mechanical tests confirmed that they are much more resistant to fracture than synthetic calcite.[10-13] Furthermore, they fracture like amorphous materials, resulting in the curved glassy surfaces typical of the so-called conchoidal fracture. Synthetic calcite crystals, grown in the presence of proteins extracted from within sea urchin spines and tests, also cleaved with conchoidal fracture, which is very different from the typical flat surfaces of cleaved crystals of the control pure calcite.[2]

During the crystal growth experiment, the proteins caused a specific change in the calcite crystal morphology, indicating that the proteins are preferentially adsorbed along planes roughly parallel to the c axis of calcite, and forming an angle of ~45° with the well developed cleavage rhombohedron {104} faces. Biochemical tests confirmed that protein was

occluded in these crystals in amounts of 0.02% by weight.[2] Preliminary mechanical tests performed by microindentation, produced stress-strain curves (Figure 1, unpublished results) indicative of increased plasticity and reduced brittleness of the crystals with occluded protein, relative to those of pure calcite.

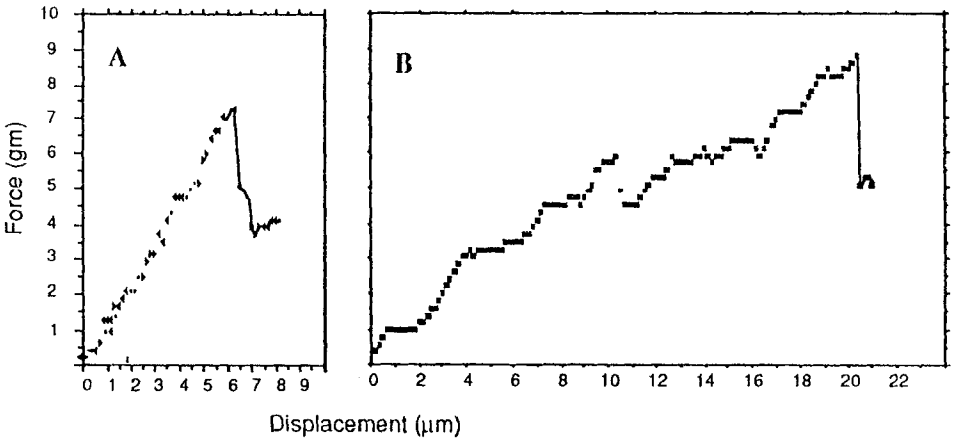


FIGURE 1 Applied force-displacement diagram obtained during microindentation experiments on: A) a pure synthetic calcite crystal. B) a synthetic calcite crystal containing proteins extracted from sea urchin spines. The sharp decrease corresponds to crystal fracture. Note the difference in initial slope and in the penetration of the indenter in the crystal before fracture occurs. The area under the curve is proportional to the energy absorbed by the crystal, and hence to the material toughness.

It is clear that macromolecules intercalated inside the crystal lattice are responsible for the modified mechanical properties of the crystal. The molecular mechanism of this specific incorporation is, however, not as clear. The protein molecules are several orders of magnitude larger in area than a unit cell of calcite. They are also heterogeneous in chemical nature. They cannot therefore be incorporated inside the perfectly coherent lattice in the form of a solid solution. They must reside at the boundaries between perfect domains inside the lattice, stabilizing existing imperfections or creating new ones. Because of their small amounts, it has not been possible,

to date, to directly image the macromolecules inside the crystals, but it is possible to study their influence on the crystal structure, by monitoring the parameters used to describe crystal perfection, or crystal texture.[3]

## PARAMETERS OF CRYSTAL TEXTURE: COHERENCE LENGTH AND ANGULAR SPREAD

Calcite crystals, including those with occluded proteins, are extremely well ordered crystals, and hence it is necessary to study their texture using highly collimated synchrotron X-ray radiation. Two parameters of crystal texture were measured by synchrotron X-ray diffraction peak profile analysis. The coherence length represents the average size of perfectly coherent domains between imperfections. Each crystallographic direction may have a typical coherence length, characterized by the diffraction of sets of planes perpendicular to the specific direction. The angular spread represents the extent of misalignment between domains, and may also vary in different crystallographic directions. These two parameters, together with lattice strain components, contribute to the width of the diffraction peaks in the $\omega/2\theta$ and $\omega$ modes respectively, and can be derived from them.[14] The presence of an analyzer crystal, introduced in the detector arm of the diffractometer, allows the separation of the contributions of the two parameters to the scans performed in the two modes. The coherence length can be evaluated from the width of the diffraction peak in the $\omega/2\theta$ mode by applying the Scherrer formula or modifications thereof, and the angular spread is directly represented by the width of the diffraction peak in the $\omega$ mode.

## ANISOTROPY IN CRYSTAL TEXTURE: CORRELATION BETWEEN MORPHOLOGICAL MODIFICATIONS AND SELECTIVE PROTEIN ADSORPTION

### The Model Systems - Calcium Fumarate and Calcium Malonate.

One of the fundamental indications of directed control of single crystal growth in the skeletal elements of sea urchins is the selective adsorption of protein along specific crystallographic planes. This is based on

morphological modifications of synthetic calcite crystals, grown in the presence of proteins extracted from within the biogenic element. No proof, however, existed that directly relates morphological modifications and selective protein adsorption on the same planes. It would be expected that a protein, once occluded inside the crystal following adsorption on specific surfaces, will preferentially decrease the coherence length in the direction perpendicular to the planes of intercalation, relative to other directions. This, in turn, should result in anisotropic effects on coherence length, when compared to crystals grown in the absence of protein.

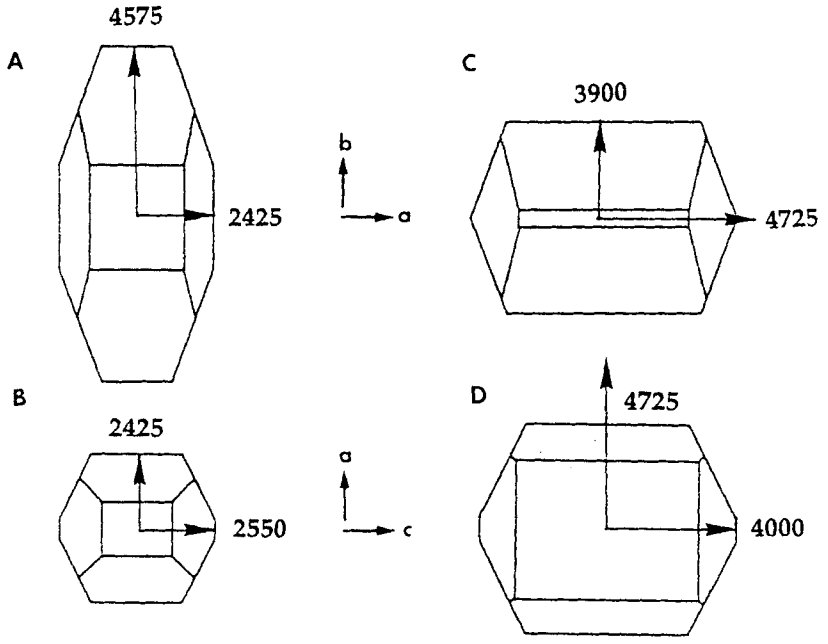


FIGURE 2 Reconstruction of the average "morphologies" of perfect domains within calcium fumarate crystals, based on the measured coherence lengths in the various directions. A) - B) a pure calcium fumarate crystal. C) - D) a calcium fumarate crystal containing proteins extracted from sea urchin spines. The proteins are intercalated along the {010} planes, perpendicular to the b axis. The measured coherence length values are reported in the main directions (arrows, Å). Note that in the protein - affected crystal the coherence length along [100] is larger than along [110], thus the former exceeds the limits of the reconstructed domain morphology.

As this concept is crucial to the argument of biological control of crystal growth, it was examined separately in two model crystal systems, namely calcium fumarate trihydrate and calcium malonate dihydrate.[15] In both these systems, morphological modifications of the crystals occur, due to the presence of protein in the growth solution. We interpreted these morphological modifications as reflecting specific adsorption of protein on crystal planes displaying at their surface structural motifs complementary to typical acidic domains on the protein.[16] We then showed that the coherence lengths for a set of crystals grown in the presence of protein, were selectively reduced in the directions perpendicular to those planes on which protein was occluded as compared to controls grown in the absence of protein (Figure 2). The correlation between protein adsorption in preferred directions revealed by morphological modifications, and anisotropic decrease in coherence length in the same directions, was thus established in model systems, allowing us to better proceed with analyses of biogenic calcite crystals.

Biogenic Single Calcite Crystals.

*Sea Urchin Larval Spicules and Adult Spines.* In the larval stage of sea urchin development, the organism is supported by a skeleton formed of two spicules, each one of which is a single crystal of calcite. In the fully developed spicule of *Paracentrotus lividus* the crystal assumes a shape roughly elongated along the c axis (Figure 3A).[17] Although there is a great similarity between the mode of growth of the larval spicule and of the adult spines, the elements are distinct, having different shape and size (Figure 3A,B). The adult and larval elements do, however, have very similar crystal textures. The values of angular spread and coherence length, averaged over all the reflections collected in different crystallographic directions, fall in the same range.[3] This range appears to be typical of the taxon (see below). When the coherence length values in the different crystallographic directions are compared, a selective decrease is observed for the planes parallel to the c axis, relative to the perpendicular direction (Figure 3A,B).[3] We note that synthetic calcite crystals do not display substantial anisotropy in coherence length in the whole diffraction sphere.[15] This observed anisotropic behavior is in full agreement with the selective intercalation of protein along planes roughly parallel to the c axis, proposed on the basis of morphological modifications in synthetic crystals (Figure 4B).

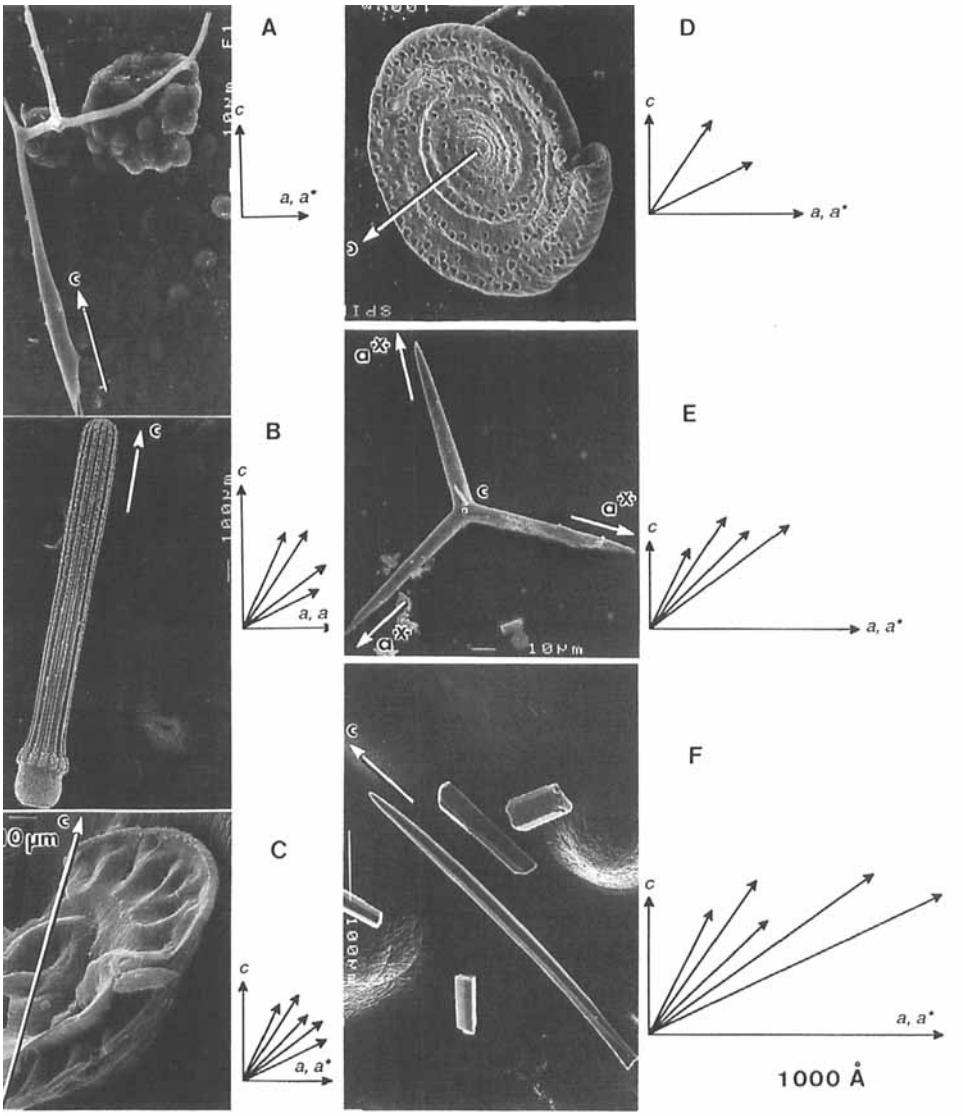


FIGURE 3 Scanning electron micrographs of the biogenic elements and the respective coherence length values. The c axis direction is indicated by arrows in the micrographs. In the diagrams, the arrow length is proportional to the measured coherence lengths. The directions correspond to the vectors of the diffracting planes. A) - B) Larval spicule and newly formed spine from the sea urchin *Paracentrotus lividus*. C) Shell of the foraminifer *Patellina corrugata*, ventral side. The coil can be seen through the broken portion in front. D) Shell of the foraminifer *Spirillina sp.* E) Triradiate calcareous sponge spicule (species unknown). F) Prism isolated from the polycrystalline prismatic shell layer of the mollusk *Atrina serrata*.

Furthermore, slow epitaxial overgrowth of new calcite crystals on cleaned spines, results in decoration of the biogenic elements with newly grown crystals, that also express stepped faces roughly parallel to the c axis.[18] The development of these faces was attributed to adsorption of protein leaking out from the partially etched biogenic element and then interacting from solution with the overgrowing crystal. This process should occur with the protein in a state as close as possible to its native structure.

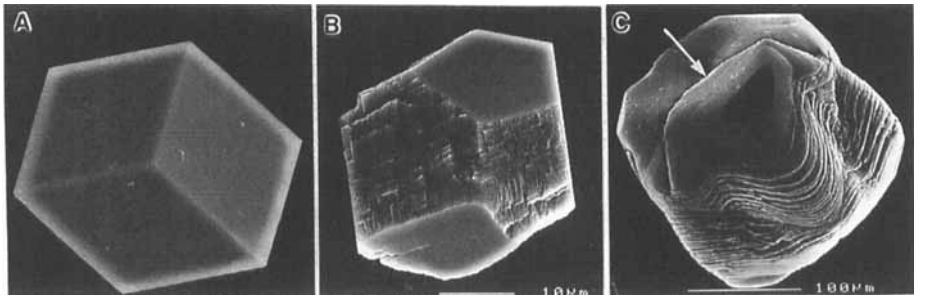


FIGURE 4 Scanning electron micrographs of synthetic calcite crystals. A) A pure calcite crystal. All faces are identical {104} faces. B) Calcite crystal grown in the presence of proteins extracted from sea urchin spines. The smooth faces are {104} and the rough ones are {01*l*}. C) Calcite crystal grown in the presence of proteins extracted from within calcareous sponge spicules. The large face is (001) (marked by an arrow). The lateral rough surface is due to EDTA adsorption.

*The Foraminifera - Spirillina and Patellina.* The Foraminifera are a diverse and abundant group of marine protozoans that generally form calcitic shells composed of arrays of crystals. *Patellina* and *Spirillina*, however, form their entire shell out of one single crystal of calcite.[19] The shell of *Patellina* is an elevated coiled tube with its c axis parallel to the base of the cone (Figure 3C). In contrast, the test of *Spirillina* is a flat coiled tube with its c axis perpendicular to the coil (Figure 3D). Both crystals have a relatively large average angular spread (0.20-0.25°); not surprising if the complex morphologies of these unusual single crystals are taken into account. The organism must somehow keep the unit cells in register over a huge distance, relative to the net volume of the crystalline phase. The anisotropy in coherence length is also extremely interesting. The coherence length of

the *Spirillina* shell is relatively large in the ab plane of calcite, but is selectively shortened in the c direction which is the morphological axis of the element (Figure 3D). In contrast, the c axis of the *Patellina* shell is not parallel to any symmetry axis of the macroscopic morphology, and does not show any anisotropy in the average coherence length (Figure 3C).[3]

*Triradiate Sponge Spicules.* Sponges are relatively primitive animals, that may develop either siliceous or calcitic spicules (or no spicules at all) to provide support for their soft tissues. In some calcisponges, many spicules of different morphologies are distributed in the tissue. Each spicule is a single crystal of calcite.[20] In some species, the spicules have a typical triradiate morphology that develops in agreement with the intrinsic symmetry of calcite, that is the c axis of calcite is perpendicular to the triradiate spicule, and each spicule arm develops along the a* axis (Figure 3E). Slow epitaxial overgrowth of calcite on cleaned spicules resulted in decoration of the biogenic elements with crystals that surprisingly developed the unusual ionic {001} face, besides other faces (Figure 5).[18] Subsequent growth of calcite from solutions containing the macromolecules extracted from the spicules after their complete dissolution, also resulted in crystals with well developed {001} faces (Figure 4C).

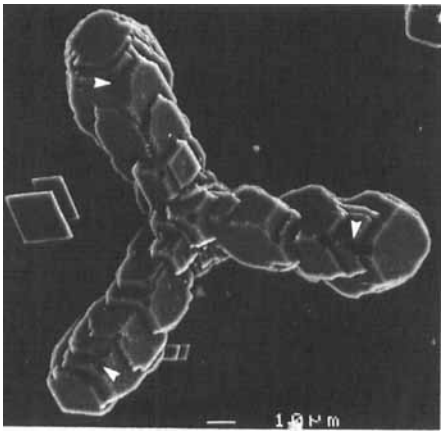

FIGURE 5 Overgrowth of calcite crystals on a calcitic triradiate sponge spicule of *Clathrina coriacea* (compare to Figure 3E). Note the development of {001} faces (some are marked by arrows), due to adsorption of proteins leaking out of the element, onto the newly forming crystals.

These results indicate preferential intercalation of proteins along the planes perpendicular to the c axis, an unusual new observation. In terms of angular spread, the triradiate spicules are fairly well ordered (0.045-0.070°), consistent with the more compact nature of the elements. The average coherence lengths, however, are also relatively high. Most interestingly, a typical anisotropy is again observed in which the coherence length is selectively shorter along the c axis (Figure 3E), which agrees well with the data on crystal growth (Figure 4C).

*Single Prisms from Mollusk Shell Prismatic Layer.* The shells of bivalves are composed of an assemblage of crystals, each of which is embedded in an organic matrix. In the mollusk *Atrina*, the prisms that make up the outer shell layer are relatively large single crystals of calcite.[21] The c axis is parallel to the long axis of the prism (Figure 3F). Epitaxial overgrowth of new calcite crystals on the cleaned dispersed prisms resulted in the newly-formed crystals developing faces both perpendicular and roughly parallel to the c axis.[18] Recently, two distinct protein fractions were isolated from the ensemble of proteins extracted from within the prisms, each one of which appears to be responsible for the development of one face type.[22] Thus, one protein fraction is intercalated on planes parallel to the long axis of the prism, and the other in the perpendicular direction. It is interesting to note that the so-called "growth lines", are on the {001} planes and may well be due to occluded organic material, as suggested by light etching of the prisms with acid.[23] Consistent with these observations, we observed an anisotropy in coherence length with the values along the c direction being selectively shortened (Figure 3F). The angular spread of isolated prisms is very small (0.02-0.03°), and the coherence lengths are high, both average values being closer to pure calcite crystals than those of all the other biogenic elements.[3]

## DISCUSSION

Some general conclusions may be drawn. The first observation is that a plot of the average coherence lengths and angular spreads for all the reflections shows that the taxonomic groups studied are concentrated in defined domains, typical of each element type.[3] This observation clearly shows that organisms control the textural parameters of their skeletal elements, and

each taxonomic group studied does it in its own way. A good illustration of this is that sea urchin adult spines and larval spicules have very similar values, even though they are very different with respect to crystal size and shape. In terms of the hypothesis formulated on the use of protein intercalation to control fracture behavior, we note that the elements that function as a polycrystalline ensemble embedded in a matrix (e.g. mollusk shell prisms) are texturally more perfect than those in which the single crystal is a distinct skeletal unit. This raises the possibility that textural control in the former tissues is less crucial, as the crystals themselves are embedded in an organic matrix. This latter structural organization provides additional mechanical benefits.

A specific anisotropy was observed for all the biogenic elements, excluding the foraminifer *Patellina*. The sea urchin adult spine and larval spicule displayed similar properties, with the coherence length in the direction parallel to the c axis being larger than that in the perpendicular direction. The mollusk prisms, triradiate sponge spicules and the foraminifer *Spirillina* showed an opposite trend, with their coherence lengths in the direction parallel to the c axis being shorter than those in the perpendicular directions. In each case, mollusk prisms excluded, the anisotropic behavior of the coherence length is in agreement with the macroscopic morphology of the element. This strengthens the notion that intercalated proteins are used to modulate the extent of crystal growth, selectively inhibiting it in specific directions where growth must be limited. In the mollusk prisms this trend is not respected, because the macroscopic morphology shows elongation in the c direction. Here there is an interesting interplay between the influence on final morphology of the crystals growing in a preformed matrix and that of extracted proteins interacting from solution with the growing calcite crystal. The exact mode of crystal growth is not known, but etching and pyrolysis of the prisms indicate that the proteins are indeed concentrated in layers perpendicular to the c axis.[23] In the foraminifer *Patellina*, on the other hand, the correlation between morphology and crystallographic axes orientation does not respect the crystallographic symmetry of calcite, such that a prediction of the crystal growth modulation effects is not trivial. Finally, the information deduced from the epitaxial overgrowth experiments and the synthetic calcite growth in the presence of extracted proteins are in perfect agreement with intercalation of protein in the same directions, as indicated by the coherence

length measurements. This is true for the sea urchin spine, for the triradiate sponge spicules and for the mollusk prisms.

Control of texture appears to be not only widespread, but much more sophisticated than initially expected. Modulation of the fracture properties of the skeletal materials is but one of the parameters under control. The controlled introduction of different ions, the definition of crystal orientation, and, most important, the total control of the microenvironment at the growth site are all biological strategies for controlling these processes. We note that synthetic calcite crystals grown from solution in the presence of proteins extracted from the biogenic elements, do not have the same textural characteristics as the original biogenic crystals. In particular, they fail to show anisotropy in coherence length in the expected directions. Clearly the degree of control achieved by organisms requires a complex and sophisticated system. We still have much to learn!



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