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Coexistence of Amorphous and Crystalline Calcium Carbonate in Skeletal Tissues

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We describe a new type of composite skeletal tissues in which calcite and stabilized amorphous calcium carbonate (ACC) coexist in well-defined domains. The organisms that form such structures are widely separated in the animal kingdom phylogenetic tree: calcareous sponges and ascidians. This paper compares the microstructures of their composite skeletal elements: The triradiate spicules from the sponge *Clathrina* are composed of a core of calcite embedded in a thick layer of ACC and covered by a thin calcitic envelope; the tunic spicules from the ascidian *Pyura pachydermatina* are composed of a core of ACC enveloped by an insoluble organic sheath and covered by a thick calcitic layer. We compare and contrast the macromolecules associated with different amorphous and crystalline phases and their ability to induce the formation of stabilized ACC in vitro.

Keywords Amorphous Calcium Carbonate, Ascidians, Biomineralization, Calcareous Sponges, Calcite.

INTRODUCTION

Amorphous calcium carbonate (ACC) is unstable thermodynamically and kinetically under ambient conditions [1]. Because of ACC's instability and ease of dissolution, it is used by various organisms in different taxonomic groups as a temporary storage site for the calcium and carbonate ions [2]. Our attention was drawn to the fact that this phase is also used in nature for structural purposes. Stable biologically produced ACC is known to be formed by members of the plant kingdom, Cyanobacteria, Mollusca, Arthropoda, and Chordata [2–5]. Our careful study of some skeletal elements that for 100 years were believed to be composed of a single calcite crystal, opened up a new world—a diversity of composite skeletal elements containing crystalline calcite in one layer and stabilized amorphous calcium carbonate in another; a situation that is technically not easy to detect [6, 7]. An organism that uses the unstable mineral as a skeletal material should provide a permanent means to stabilize this phase.

Macromolecules found within biogenic calcium carbonate phases are the key components controlling their crystalline properties, such as morphology of biogenic single calcite crystals [8] and their mechanical [9] and textural [10] characteristics. We have also suggested that at the opposite extreme of protein control over mineral formation, specialized macromolecules appear to be involved in the inhibition of crystallization and stabilization of unstable ACC [6], or in the formation of ACC, which is intimately associated with calcite and acts as a precursor phase of the latter [11, 12]. We examined skeletal elements that contain amorphous $CaCO_3$ and calcite adjacent to each other. We show that macromolecular extracts from biogenic amorphous $CaCO_3$ skeletons from completely different taxonomic phyla are composed of glycoproteins rich in GlX, Ser, and Thr. All inhibit the crystallization process and induce precipitation of a stabilized amorphous phase in vitro. In contrast, the proteins extracted from the crystalline calcitic phase from the same elements are AsX-rich and induce the formation of calcite crystals with modified morphologies.

RESULTS AND DISCUSSION

Exoskeletons of the solitary ascidian *Pyura pachydermatina* (Urochordata, Ascidiacea) and calcareous sponge *Clathrina* (Porifera) were isolated and cleaned as described previously [6]. The skeletal spicules are shown in Figure 1. A detailed study of spicules microstructure and mineral identity was performed by electron microscopy, overgrowth, and etching experiments (Figures 2 and 3), infrared spectroscopy (FTIR) (Figure 4) and X-ray diffraction (XRD) (Figure 5).

The antler-shaped body spicules formed by *P. pachydermatina* are composed entirely of stabilized amorphous calcium carbonate (Figure 1A). The amorphous character of these spicules was confirmed by FTIR (Figure 4C) and synchrotron powder

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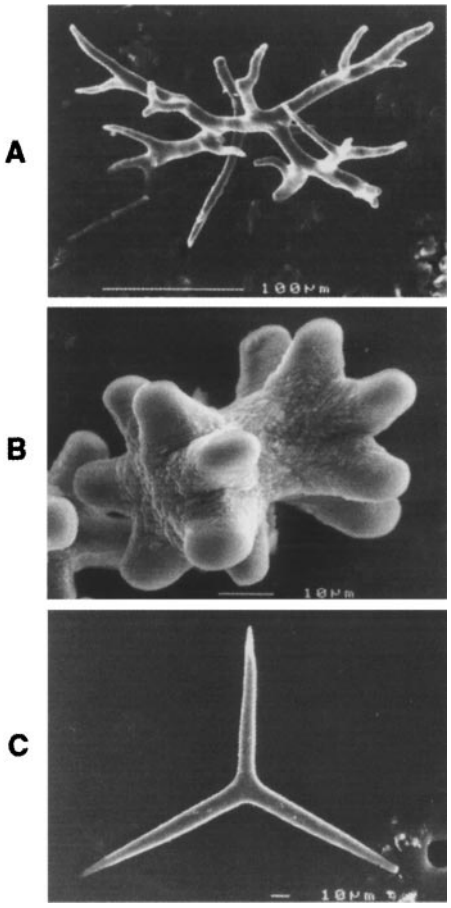

Figure 1. (A) Body (antler) spicule formed by the ascidian *P. pachydermatina*. (B) Tunic (dogbone) spicule formed by *P. pachydermatina*. (C) Triradiate spicule formed by the calcareous sponge *Clathrina*.

XRD (Figure 5C). The tunic spicules produced by the same organism were thought to be composed of a single calcite crystal [5]. They have a typical dogbone shape with rough surfaces that show a characteristic calcitic texture exhibiting the $\{011\}$ steps (Figure 1B). Indeed, the FTIR spectrum (Figure 4B) and powder XRD of the intact spicules demonstrated only the characteristic, slightly broadened calcitic peaks. Synthetic calcite crystals grown epitaxially on the spicule surfaces [13] were coaligned in the *c*-axis direction but slightly misaligned in the *a*, *b*-plane, suggesting that the underlying biogenic material is a polycrystalline calcite with the preferred *c*-axis orientation (Figure 2A).

Although the surfaces of dry, freshly sectioned spicules are smooth and show no indication of phase boundaries (Figure 2B), their exposure to water for 3–5 hr results in selective etching of the inner part of the spicules (Figure 2C). Upon heating in a drop of water, the core recrystallizes into calcite, which is oriented epitaxially with the calcitic envelope (in Figure 2E, note the three-fold symmetry of the recrystallized core). These results suggest that the inner part is composed of ACC. The selective removal of the ACC core in water reveals an insoluble organic sheath between the outer calcitic layer and the inner amorphous core (Figure 2E). Bleaching in a 2.5% NaOCl solution for 1 day completely dissolves the ACC core and the surrounding organic sheath (Figure 2D). The ACC cores (Figure 2F) can be quantitatively fractionated by differential density sedimentation. Spicules were suspended in bromobenzene (density 1.497 g/ml) and were allowed to precipitate for 10 min. After sedimentation, the supernatant with the suspended lightest spicule fraction was removed, washed, dried, and characterized. The FTIR spectrum of this fraction confirms that it is composed mostly of ACC (Figure 4D). The weight fraction of the ACC in mature spicules is ∼15–20%.

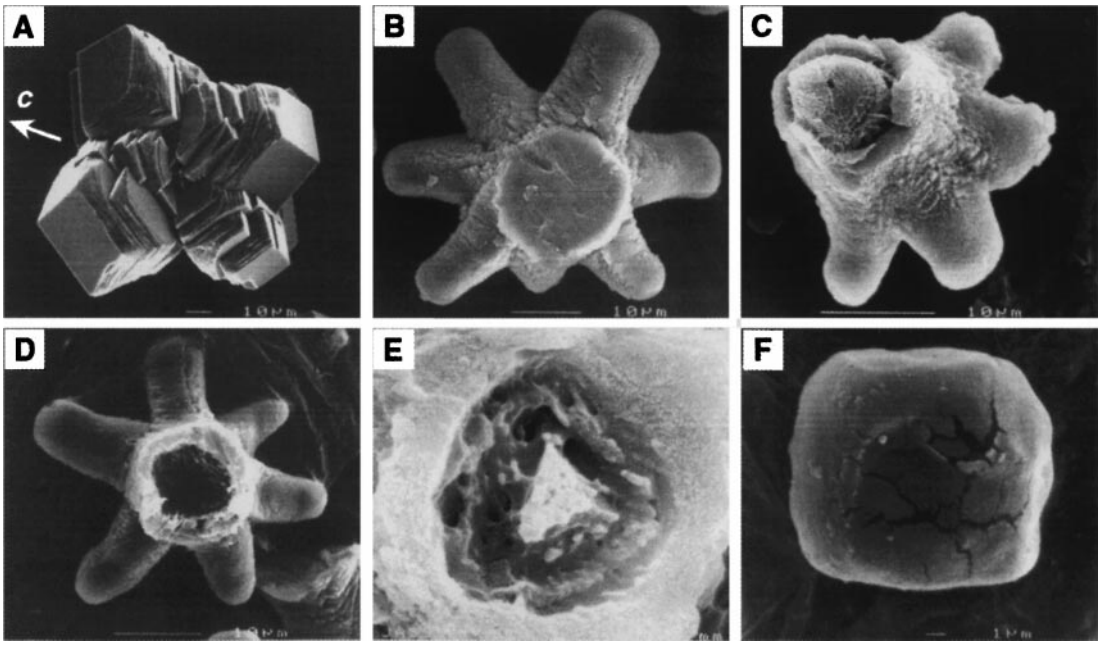

Figure 2. Chemical characterization of ascidian dogbone spicules shows that they are composite structures with the combination of two mineral phases: the ACC core and the calcitic envelope separated by an organic sheath. (A) Overgrowth of the spicule surface with synthetic calcite crystals. (B) Untreated cross-section. (C) Etching of the inner part of the sectioned spicules in water. (D) Removal of the core in bleach. (E) Recrystallization of the core upon heating. (F) ACC core isolated by differential sedimentation.

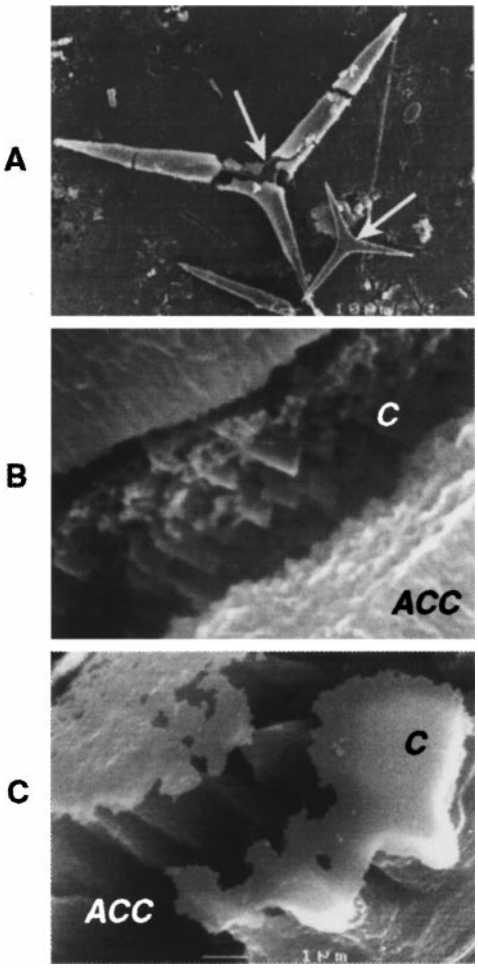

Figure 3. Chemical treatment of sponge triradiate spicules reveals the combination of two mineral phases: the calcitic core and the ACC envelope covered with a thin calcitic layer. (A) Heated spicule clearly shows the dense calcitic cores (indicated by arrows) and an outer layer of ACC. High magnification of partially KOH-etched spicule reveals shapeless etch pits in the ACC layer, regular crystalline etch figures on the surface of the calcitic core (B) and a thin calcitic outer sheath (C).

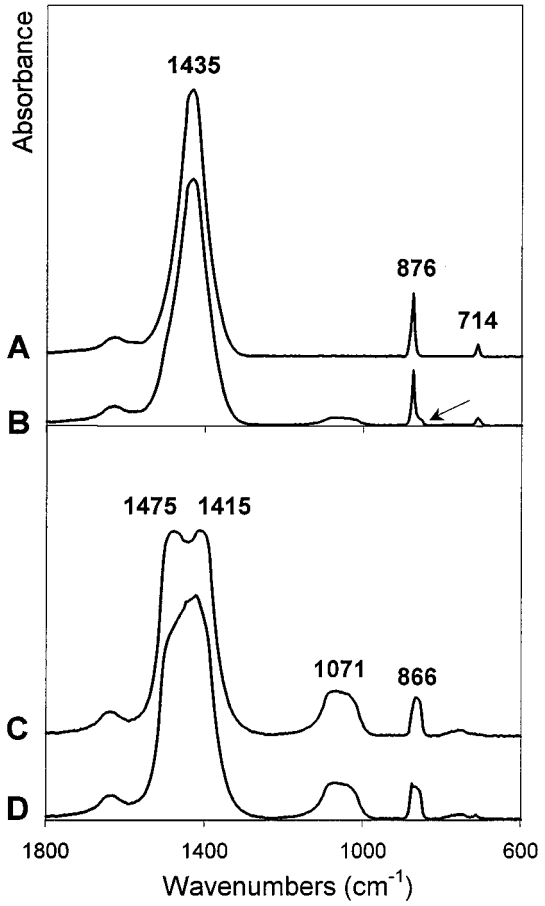

Figure 4. (A) Infrared spectra of pure calcite; (B) intact ascidian dogbone spicules; (C) intact ascidian antler spicules; (D) ACC core of dogbone spicules separated by differential density sedimentation.

Triradiate spicules formed by *Clathrina* also have been reported as an example of single crystalline calcitic skeletal elements (Figure 1C). Our etching experiments showed that the spicules are, in fact, composite structures with a calcitic core embedded into a thick amorphous layer, which is, in turn, covered with a thin calcitic envelope (Figure 3) [6]. Both phases are clearly seen in the synchrotron XRD spectrum (Figure 5B). From the comparison of the intensities of the different crystallographic peaks with those of an equal volume of pure calcite (Figure 5A), the amount of crystalline material in the *Clathrina* spicules was found to be ∼20%. On the basis of the above observations, we deduced that ascidian dogbone spicules and sponge triradiate spicules are examples of a new type of a composite tissue, in which amorphous and crystalline calcium carbonates form one skeletal structure where they coexist in well-defined domains.

To address the question of the involvement of specialized proteins in the formation of these composite structures, we extracted macromolecules associated with the calcitic and ACC phases by differential dissolution. Crushed spicules were suspended in DDW for 3–4 days and placed on a rocking table in a cold room (4–5°C). This treatment resulted in the differential, quantitative dissolution of the ACC phase and release of the associated proteins. The residual calcitic material was dissolved in HCl to yield macromolecules associated with the calcitic phase. The fractions obtained were characterized by amino acid analysis and FTIR. The protein content in the ACC phases is characteristically higher than in the calcitic layers. In triradiate spicules, the protein concentration (in weight % of mineral) was 0.135 and 0.062 in the ACC layer and calcitic core, respectively. In dogbone spicules, the protein concentration was 0.049 and 0.009 in the ACC core and calcitic layer respectively. The amino acid compositions are shown in Table 1. Macromolecules extracted differentially from the calcitic layers of dogbone and triradiate spicules are AsX-rich, as is characteristic of the proteins associated with many other crystalline $CaCO_3$ skeletons [1, 14]. Macromolecules extracted differentially from the ACC

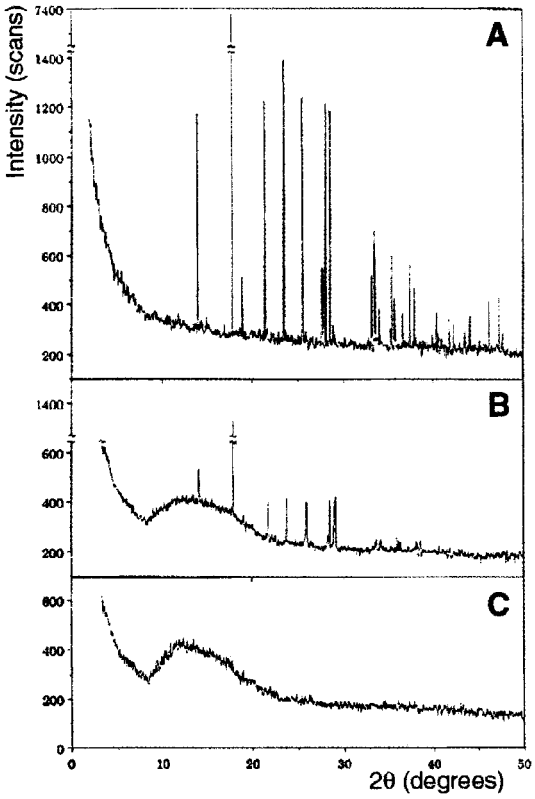

Figure 5. (A) Synchrotron powder X-ray diffraction spectra of pure calcite; (B) intact triradiate sponge spicules; (C) ascidian antler spicules.

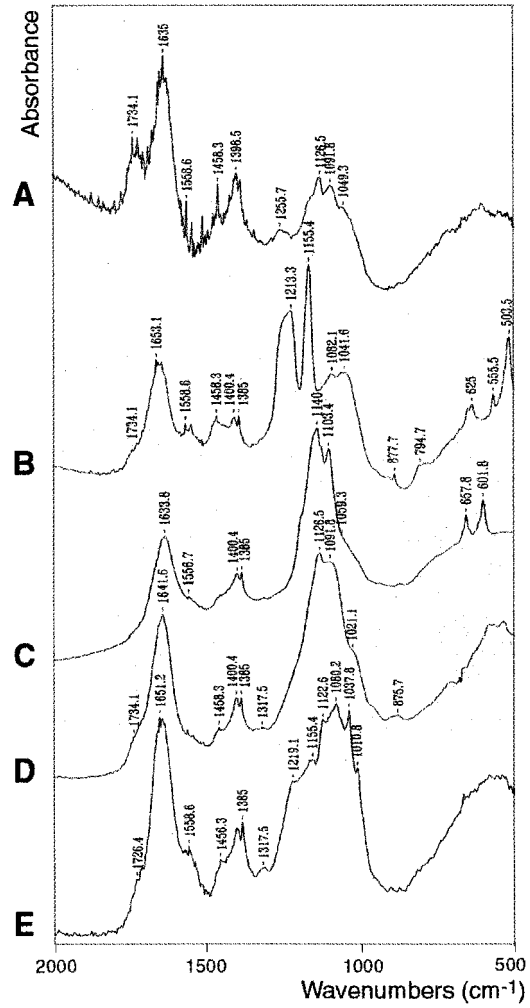

Figure 6. Infrared spectra of extracted macromolecules: (A) from the calcitic layer of dogbone spicules; (B) insoluble organic sheath in dogbone spicules; (C) from the ACC core of dogbone spicules; (D) from the ACC of antler spicules; (E) from the ACC layer of the sponge triradiate spicules. Note the broad sugar bands at around 1100 cm^{-1} in (C) and (E) and the lipid band at around 1735 cm^{-1} in (A), (B), (D), and (E).

layers of dogbone and triradiate spicules and total macromolecular extract from antler spicules are quite different from those associated with biogenic calcite. They are all typically rich in hydroxyamino acids (serine and threonine), with the GlX content being characteristically higher than that of AsX. FTIR spectra show that the macromolecules associated with the ACC phase also are heavily glycosylated, while those extracted from calcitic layers are not (Figure 6).

The proteins associated with the two calcium carbonate phases were probed for their ability to affect the formation of $CaCO_3$ in vitro. Figure 7 shows calcium carbonate precipitates formed from the solutions saturated with respect to $CaCO_3$ in the presence of different macromolecular extracts.

The formation of spherical particles of stabilized synthetic ACC was observed with the addition of macromolecules extracted from the stable amorphous biomaterial of the antler spicules (Figure 7A) and ACC layer of triradiate spicules (Figure 7B). The amorphous character of these synthetic $CaCO_3$ phases was deduced from FTIR and electron diffraction [6, 7]. Macromolecules extracted from the ACC core of the dogbone spicules induced complete inhibition of crystallization. We did not, however, detect any significant amount of ACC. This observation does not rule out the formation of ACC, which could presumably dissolve during the experiment in the calcium chloride solution, as does its partially stabilized biogenic original phase. The protein content of synthetic ACC was determined from amino acid analysis of the dissolved precipitates and estimated to be 0.1–0.15 wt% of mineral. These macromolecules appear further enriched in GlX and sugars. We concluded, therefore, that specialized glycosylated proteins, rich in GlX, Ser, and Thr, are involved in the stabilization of the otherwise thermodynamically and kinetically very unstable phase, amorphous $CaCO_3$.

The addition of macromolecules extracted from the calcitic layer of dogbone spicules to solutions facilitated the crystallization process compared with the control experiments carried out without any additives. The crystals formed are calcite with modified morphology. In addition to the normal $\{104\}$ cleavage planes of pure calcite, these crystals exhibited stepped faces slightly oblique to the c-axis, indexed as $\{01l\}$ ($l \sim 1.5$) (Figure 7C). This modified crystal morphology resembles the

TABLE 1
Amino acid composition of the proteins extracted from the ascidian and sponge spicules.

Amino acid	Antler spicules intact	Sponge spicules ACC layer	Sponge spicules calcitic core	Dogbone spicules ACC core	Dogbone spicules calcitic layer	Dogbone spicules organic sheath
Asx	**7.8**	**7.2**	**28.5**	**7.1**	**18.8**	**11.7**
Glx	**16.8**	**17.7**	**14.3**	**17.2**	**8.0**	**11.4**
Ser	**18.0**	**19.2**	**8.8**	**11.2**	**6.8**	**7.3**
Thr	**4.6**	**4.3**	**3.5**	**12.2**	**4.6**	**13.2**
Gly	19.6	19.9	10.8	12.6	20.8	11.2
Ala	8.0	8.4	10.5	5.5	6.5	7.3
Val	3.5	3.5	3.5	6.0	4.6	6.2
Leu	3.5	2.3	2.9	5.3	7.1	5.6
Ile	2.4	1.7	2.0	3.0	1.2	3.7
Arg	2.3	1.1	2.0	8.9	3.4	3.7
Lys	3.0	4.1	1.7	4.4	4.0	7.9
His	3.1	3.2	1.1	–	0.8	1.8
Phe	1.7	1.0	2.3	3.3	1.2	3.7
Tyr	1.8	0.8	1.6	0.4	2.2	0.6
Cys	0.1	–	0.2	1.6	7.8	1.8
Met	0.6	–	2.1	–	1.2	0.9
Pro	3.2	5.4	4.2	1.2	1.0	2.1

family of calcite faces on the surfaces of the dogbone spicules (Figure 1B) and those in the slow overgrowth experiments (Figure 2A). Calcite crystals flattened in the *c*-axis direction (well-developed $\{001\}$ crystallographic face is indicated by an arrow) were formed in the presence of the macromolecular extract from the calcitic cores of sponge spicules (Figure 7D). This modified crystal morphology correlates well with the original triradiates, which are restricted in growth in the *c*-axis direction and develop in the a, b-plane (Figure 1C). The observed specific changes in the morphology of synthetic calcite crystals imply that intracrystalline macromolecules are involved in the formation and shaping of the biogenic calcitic phase.

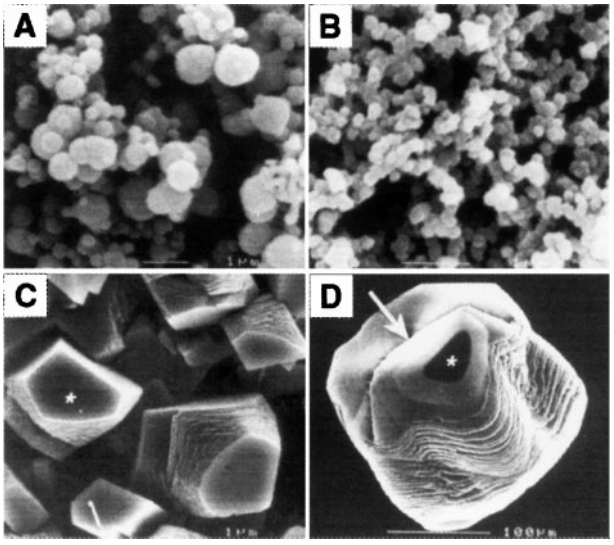

Figure 7. Calcium carbonate grown in vitro with the addition of specialized proteins. Stabilization of ACC in vitro can be achieved using macromolecules from the ascidian antler spicules (A) and from the amorphous layer of the sponge triradiate spicules (B). Calcite crystals with modified morphologies are formed after the addition of macromolecules from the calcitic layer of the ascidian dogbone spicules (C) and from the calcitic core of the sponge triradiate spicules. $*$ = the $\{104\}$ faces of calcite.

CONCLUSION

We described a new type of composite skeletal elements in which crystalline and amorphous calcium carbonates are juxtaposed in a sophisticated microstructure. We suggested that their formation is apparently controlled by specialized macromolecules, which show similar activity in vitro. We can only speculate at this moment that composite structures in which amorphous and crystalline phases coexist apparently endow the biogenic material with advantageous mechanical properties. We suspect that such structures are a lot more widespread and could be easily overlooked due to the difficulties involved in the detection of ACC when it is associated with crystalline polymorphs.

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