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Biomineralization in living hypercalcified demosponges: Toward a shared mechanism?

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ABSTRACT

Massive skeletons of living hypercalcified sponges, representative organisms of basal Metazoa, are uncommon models to improve our knowledge on biomineralization mechanisms and their possible evolution through time. Eight living species belonging to various orders of Demospongiae were selected for a comparative mineralogical characterization of their aragonitic or calcitic massive basal skeleton. The latter was prepared for scanning and transmission electron microscopy (SEM and TEM), selected-area electron diffraction (SAED) and X-ray diffraction (XRD) analyses. SEM results indicated distinctive macro- and micro-structural organizations of the skeleton for each species, likely resulting from a genetically dictated variation in the control exerted on their formation. However, most skeletons investigated shared submicron to nano-scale morphological and crystallographical patterns: (1) single-crystal fibers and bundles were composed of 20 to 100 nm large submicronic grains, the smallest structural units, (2) nano-scale likely organic material occurred both within and between these structural units, (3) {1 1 0} micro-twin planes were observed along aragonitic fibers, and (4) individual fibers or small bundles protruded from the external growing surface of skeletons. This comparative mineralogical study of phylogenetically distant species brings further evidences to recent biomineralization models already proposed for sponges, corals, mollusks, brachiopods and echinoderms and to the hypothesis of the universal and ancestral character of such mechanisms in Metazoa.

1. Introduction

Among more than 8500 valid Recent sponge species known today (van Soest et al., 2012), only a few ones produce a massive basal skeleton of calcium carbonate, in addition to a siliceous or calcareous spicule framework. These so-called hypercalcified sponges, luxuriant reef-builders of late Paleozoic and Mesozoic eras, were considered to be extinct until a handful of living species were rediscovered almost half a century ago. Scuba diving and submersible explorations allowed to extend our knowledge on these coralline sponges with the discovery of nearly twenty new living species found almost exclusively in cryptic or deep habitats in tropical seas and the Mediterranean (Hartman, 1969, 1979; Hartman and Goreau, 1970, 1975, 1976; Vacelet, 1964, 1970; Vacelet and Lévi, 1958; Willenz and Pomponi, 1996). Recent hypercalcified sponges form a polyphyletic group with species belonging to various orders of both Calcarea and Demospongiae. The occurrence of a massive calcareous basal skeleton in these living forms was considered as an archaic character that would have appeared in most Paleozoic and Mesozoic taxa (Reitner, 1992; Vacelet, 1979, 1985). As some of their skeletal features are often analogous to their fossil relatives, Ca-carbonate biomineralization of these sponges was considered to be a conservative process maintained since million of years (Gautret et al., 1996; Lange et al., 2001; Reitner, 1992; Reitner and Engeser, 1987; Reitner et al., 1997, 2001; Vacelet, 1983; Wörheide, 1998). Although these evolutionary assumptions would need further demonstration, these unique Recent sponges represent valuable models to improve our understanding of the early evolution of Ca-carbonate biomineralization mechanisms.

Nonetheless, among skeletons of even phylogenetically closely related living hypercalcified species, highly diverse macro- and microstructures, organization grades, elemental/isotopic chemistry and associated organic macromolecules were described (see for review Reitner, 1992; Vacelet et al., 2010) leading authors to suggest different mineralization mechanisms. For instance, while most pre-

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vious investigations highlighted an extracellular biomineralization of basal skeleton in living hypercalcified sponges (Reitner and Gautret, 1996; Reitner et al., 2001; Willenz and Hartman, 1989; Gilis et al., 2012), the demosponge *Astrosclera willeyana* initiates the formation of its spherulitic basal skeleton through an intracellular pathway followed by a secondary extracellular growth phase (Lister, 1900; Wörheide, 1998; Wörheide et al., 1997). Wörheide (1998) provided a detailed description of biomineralization processes in this species combining three mechanisms: (1) small skeletal spherulites are formed within large vacuole cells (LVC) in the ectosome; (2) 15–20 μm large spherulites are further carried by a group of another cell type into the extracellular space, between the soft tissue and the growing skeleton, where they fuse together through secondary epitaxial growth; (3) a withdrawal of the soft tissue produces spaces in the lowermost-part of the skeleton cavities which are then filled by the epitaxial growth of spherulitic fibers. More recently, Jackson et al. (2010) have shown that intracellularly degraded bacteria were used as an organic matrix for controlling the formation of spherulites in the LVC like cells of *A. willeyana*. Furthermore, these authors discovered that the gene encoding for a matrix protein occurring in the calcified spherulite would be horizontally acquired by this sponge from a bacterium (Jackson et al., 2011). In *Acanthochaetetes wellsi*, the only known living hypercalcified demosponge producing a Mg-calcite basal skeleton, the extracellular mineralization of calcitic fibers would take place in four different locations of the skeleton, each involving a different biomineralization mechanism (Reitner and Gautret, 1996).

Although these previous macro- and microstructural mineralogical studies suggest a divergent evolution of biomineralization modes among living hypercalcified sponges, some recent observations at higher magnifications might contrarily indicate some shared pathways of biomineralization at lower scales. For example, 50–100 nm large grains that have been universally described as the smallest structural units in most calcium carbonate skeletons produced by metazoans (e.g. Cuif and Dauphin, 2005a,b; Cuif et al., 2008, 2011; Cusack et al., 2008; Goetz et al., 2011; Isa, 1986; Jacob et al., 2008; Pérez-Huerta et al., 2013; Robach et al., 2005; Rousseau et al., 2005; Schmahl et al., 2012a,b; Sethmann et al., 2006; Sethmann and Wörheide, 2008; Stolarski, 2003; Stolarski and Mazur, 2005; Weiner and Addadi, 2011) also occur in the three Recent hypercalcified demosponges species *Vaceletia crypta*, *A. willeyana* and *Ceratoporella nicholsoni* (Cuif et al., 2011) as well as in the skeleton of the living Mediterranean hypercalcified sponge *Petrobiona massiliana*, belonging to Calcarea (Gilis et al., 2011; Stolarski and Mazur, 2005).

These skeletal submicronic structures were shown to be in a transient amorphous state before crystallization during the formation of calcium carbonate skeleton in mollusk (Baronnet et al., 2008; Cuif et al., 2008, 2011; Jacob et al., 2008; Wehrmeister et al., 2011; Weiss et al., 2002), brachiopods (Goetz et al., 2011; Griesshaber et al., 2009; Schmahl et al., 2012a) and echinoderms (Beniash et al., 1997; Gong et al., 2012; Killian et al., 2009; Ma et al., 2007; Politi et al., 2004, 2006, 2008; Raz et al., 2003). Crystallization would progressively propagate through those pre-assembled submicronic amorphous grains, producing micron-scale monocrystal-like structures (Baronnet et al., 2008; Cuif et al., 2008; Cusack et al., 2008; Goetz et al., 2011; Jacob et al., 2008; Killian et al., 2009; Nouet et al., 2012; Politi et al., 2008; Przeniosło et al., 2008; Schmahl et al., 2012a,b; Weiner and Addadi, 2011), a biomineralization model also suggested for calcareous sponge spicule production (Sethmann et al., 2006; Sethmann and Wörheide, 2008) and basal skeleton formation in the hypercalcified sponge *P. massiliana* (Gilis et al., 2011).

In order to validate whether this pattern of biomineralization also prevails in phylogenetically distinct hypercalcified sponges, we investigated the basal skeleton of eight recent demosponges species, from macro- to submicronic structures, by scanning and transmission electron microscopy (SEM and TEM), electron microdiffraction (SAED) and X-ray diffraction analyses (XRD).

2. Materials and methods

Specimens of eight different living hypercalcified sponge species were collected by scuba diving and submersible explorations (Table 1). Samples were immediately preserved in ethanol 70° after collection. Before analyses, samples of each species were treated identically. Fragments were shortly exposed to a 10% sodium hypochlorite solution in order to remove superficial soft tissues. Skeletons were dehydrated in a graded ethanol series and stored in absolute ethanol until further treatments for scanning and transmission electron microscopy (SEM and TEM) and X-ray diffraction (XRD) analyses.

For SEM, samples were fractured, dried at 50 °C, mounted on aluminum stubs, carbon-coated and observed on a SEM JEOL JSM-6320F at 15 kV.

For TEM, small fragments were gently-crushed in a mortar and pestle. Resulting micron- or submicron-scale particles were mounted on holey carbon coated copper grids. Air-dried preparations were then observed on a JEOL 3010 TEM at 300 kV for TEM imaging and recording selected area electron diffraction (SAED) patterns. A low dose illumination during tuning of the objective focus and astigmatism correction was carefully used to reduce damages to biogenic carbonate. SAED diagrams were obtained by using a set of apertures, selecting skeleton areas with homogenous absorption/diffraction contrasts. A simple-tilt specimen holder was used since we intended to check only the crystallinity of the microstructures, not their local crystallography.

For XRD, finer powders were obtained for each species in a mortar and pestle. Powder diffraction measurements were realized using an INEL diffractometer fitted with a 120° curved position sensitive detector (CPS-120), working in transmission mode, and equipped with a Cu anticathode (λ = 15,418 Å) operated at 45 kV and 20 mA. Each sample powder was X-rayed separately in thin-walled (0.5 and 0.7 mm) capillary tubes for X-ray diffraction. For better accuracy, pure α-quartz was used as internal standard.

The basal skeleton of each species was investigated from macrostructure to submicronic structure. In order to identify skeleton growth steps, observations were systematically focused on micronic and submicronic structural units in internal mature areas of the skeleton exposed by fracturing, as well as in growing superficial layers. Selected area electron diffraction (SAED) patterns on TEM images and X-ray diffraction analysis on gently-crushed powders allowed to characterize respectively the amorphous, single- or poly-crystalline nature of the selected material in micronic to submicronic structures and to specify the crystalline phase(s) present in each basal skeleton.

3. Results

All morphological (SEM and TEM) and crystallographical (SAED and XRD) observations are presented individually for each species and summarized in Table 2.

3.1. A. wellsi

The basal skeleton of *A. wellsi* was made up of contiguous vertical tubes (calicles) 300–400 μm wide, subdivided by horizontal tabulae delineating inter-tabular spaces (Fig. 1a). Short spines sparsely ornamented walls of calicles, 50–100 μm large. The lamellar microstructure of walls, spines and tabulae were composed of

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