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Investigation of nanoorganized biomaterials of marine origin

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Abstract Naturally occurring nanoorganized biomaterials of marine origin provide an abundant source of novel bone and cartilage replacement materials, and enable the development of novel biomimetic composites. The design of novel biomaterial relies on an understanding of the organic matrices and templating structures. The aim of the present study was to investigate the composition and the properties of skeletal structures of marine sponge (*Verongula gigantea*) and octocorals (*Isidella* sp.) in particular by using instrumental analytical (i.e. electron transmission and scanning microscopic methods, vibrational spectroscopies) methods. Modern gentle demineralization techniques were used. It was shown, that the demosponge *V. gigantea* has much potential as a biomaterial due to the multilayered structure of its rigid fibrous skeletons. The results of FTIR and Raman spectroscopy unambiguously showed that all specimens of the investigated sponge have α-chitin as the main skeletal component. Nano-crystalline aragonite was isolated and identified in *V. gigantea*, a sponge usually described as lacking a mineral skeleton. Bamboo corals of the Isididae family were additionally investigated. An inorganic component within the deep-sea octocoral *Isidella* sp. could be clearly identified as calcite by using Raman spectroscopy. The organic part was identified as a nanoorganized fibrillar proteinaceous matrix with acidic properties.

1. Introduction

Naturally occurring nanoorganized biomaterials of marine origin provide an abundant source of novel bone and cartilage replacement materials (Green et al., 2002; Green, 2008), and enable the development of novel biomimetic composites.

Sponges are fascinating research objects because of the hierarchical organization of their fibrous skeletons (Demospongiae) and mineralized spicules, which contain amorphous silica (Demospongiae and Hexactinellida) or calcium carbonate (Calcarea). Thus, skeletal formations of sponges are examples of natural rigid glass-based or calcium carbonate based composites. Sponges are presently gaining increased scientific attention because of their secondary metabolites and their possible biotechnological applications. The well known biotechnological potential of marine sponges can be seen as a goldmine to chemists and pharmacologists: unique and innovative structures have been discovered with cytotoxic, antifouling, antitumoral, antibiotic, antiviral,

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cytoprotective, enzyme-inhibitory, anti-inflammatory and anti-Alzheimer applications (Faulkner, 2001).

Recently, we showed that chitin is present as a structural component in skeletons of both poriferan classes, Hexactinellida and Demospongia (Ehrlich et al., 2007a,b). This most intriguing finding has led us to a better understanding of the biomimetic potential of marine sponges and gives a fresh impulse to the search for new sponge specimens. Because fiber skeletons of marine demosponges (*Spongia* sp.) have recently been used as biomimetic scaffolds for human osteoprogenitor cell attachment, growth and differentiation, with their specific elastomeric and bioactive properties, and potential for applications in biomedicine and material sciences (Green et al., 2003), we are striving to obtain more information about structural peculiarities of the skeletal formations of sponge origin.

Natural corals have also proven to be particularly inspiring for chemists and material scientists. In particular the organic matrix within the calcified nodes is interesting from the purely scientific point of view as well as from the technological novel synthetic materials point of view. Deep-sea corals have been used as a bone substitute for more than 10 years in orthopedic, trauma, craniofacial, dental, and neurosurgeries. Corals have a structure similar to that of human bone, with a hard outer sheath and a spongy inner core. Even if coral is not used at the site of the original injury, it can be used to replace bone harvested from the patient at the donor site, making it possible to reharvest bone later at the same site if necessary. The following kinds of coral derived materials have been used previously for biomedical purposes (Ehrlich et al., 2006): coral hydroxyapatite and aragonite, coral granules, natural coral fragments, newly developed coral-composite materials and coral powders (coral calcium).

The aim of the present study was to investigate the composition and the properties of skeletal structures of marine sponge (*Verongula gigantea*) and octocorals (*Isidella* sp.) with emphasis placed on the measurements using instrumental analytical and biochemical methods.

2. Experimental

2.1. Sample preparation

V. gigantea samples were collected from the Caribbean Sea (Cuba). Isidid samples were collected from the Heceta Bank off the coast of OR, USA by P. Etnoyer. In order to elucidate the nature of the fiber components, the sponge species were demineralized by alkali treatment in 2.5 M NaOH for 7 days under thermostatic conditions (37 °C) as detailed described previously (Ehrlich et al., 2007a,b). The decalcification of Isidid octocoral samples was carried out by using Osteosoft (Merck) solution (Ehrlich et al., 2006).

2.2. Instrumental analytical methods

FTIR spectra of the purified samples were recorded with a Perkin–Elmer FTIR Spectrometer Spectrum 2000, equipped with an AutoImage Microscope using the FT-IRRAS technique (Fourier Transform Infrared Reflection Absorption Spectroscopy). Raman spectroscopy was made on a FT-Raman spectrometer Bruker RFS100/s by using Nd-YAG excitation at 1064 nm.

The ultrastructural morphology of the coral surface, of its axial internodes and of the node channels was characterized by scanning electron microscopy (SEM) on an ESEM XL 30, Phillips, on a SEM LEO DSM 982 Gemini and by transmission electron microscopy (TEM) on a Zeiss EM 912. Energy dispersive X-ray microanalysis (EDX) was carried out using the same instruments. Samples were sputter-coated with a thin layer of gold (Sputtercoater S 150 B, Edwards). The cuprolinic blue staining of the coral organic matrix for the TEM study was done as reported previously (Ehrlich et al., 2006).

3. Results and discussion

3.1. Sponges

Species of the demosponge *Verongula* display an elaborate honeycomb-like surface architecture (Fig. 1).

This architecture is built of well organized superficial elements of the skeleton (Erwin and Thacker, 2007). The fiber components supporting the ridges were detected by SEM, but can not be seen when applying standard light microscopy techniques. The fiber network reaches up to the surface and is also the template for the polygonal meshes, which can be found inside the skeleton. The SEM images presented in Fig. 2 show the cross (a) and longitudinal (b) sections of *V. gigantea* skeletal fibers. The fibers appear to be multilayered.

From previous investigations we found C, O, Br, S, Cl, I and Ca in the fibers of *V. gigantea* collected near the Bahamas (Ehrlich et al., 2003). The identification of calcium within the skeleton of *V. gigantea* is of great interest because this species has been suggested to be lacking in mineral compounds. The elemental analysis (EDX/ESEM) of the investigated *V. gigantea* fiber is shown in Fig. 3. The obtained results indicate that calcium is also present within this skeletal formation.

In order to obtain information about the form in which calcium-based compounds are found in the fiber, we used a demineralization procedure as described previously (Ehrlich et al., 2007a). The demineralization is a crucial step for structural investigations as well as for the exploration of the biomimetic potential of biocomposites since the analysis of the organic matrix embedded in the materials usually requires the dissolution of the mineral phases (Ehrlich et al., 2008, 2009). The treatment of the samples by 2.5 N NaOH leads to the depigmentation of the

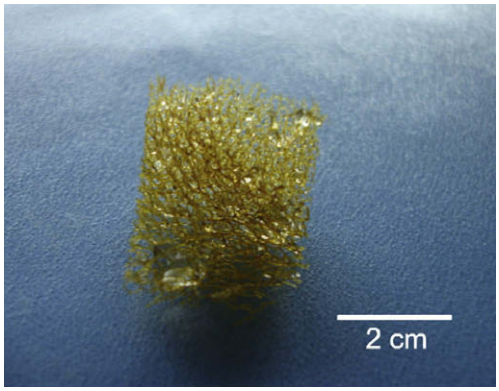

Figure 1 Fragment of the honeycomb-like skeleton of *V. gigantea*.

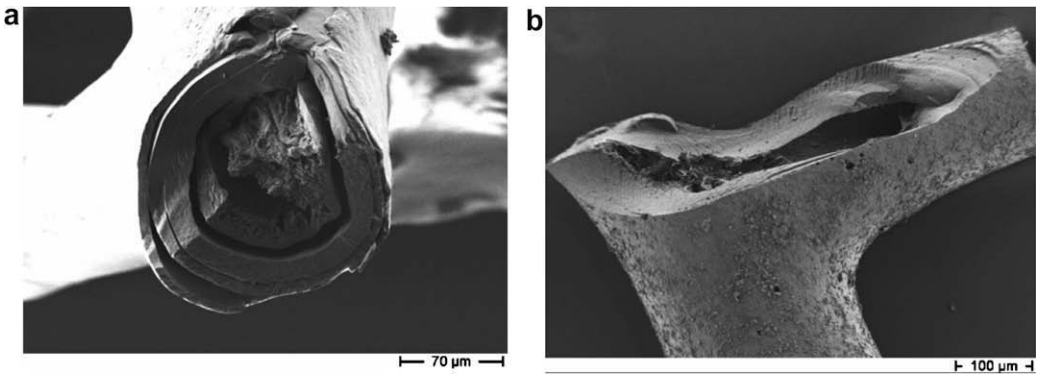

Figure 2 SEM images of the skeletal fiber of *V. gigantea*. Cross section (a) and longitudinal section (b) show the characteristic multilayered organization of the fibers.

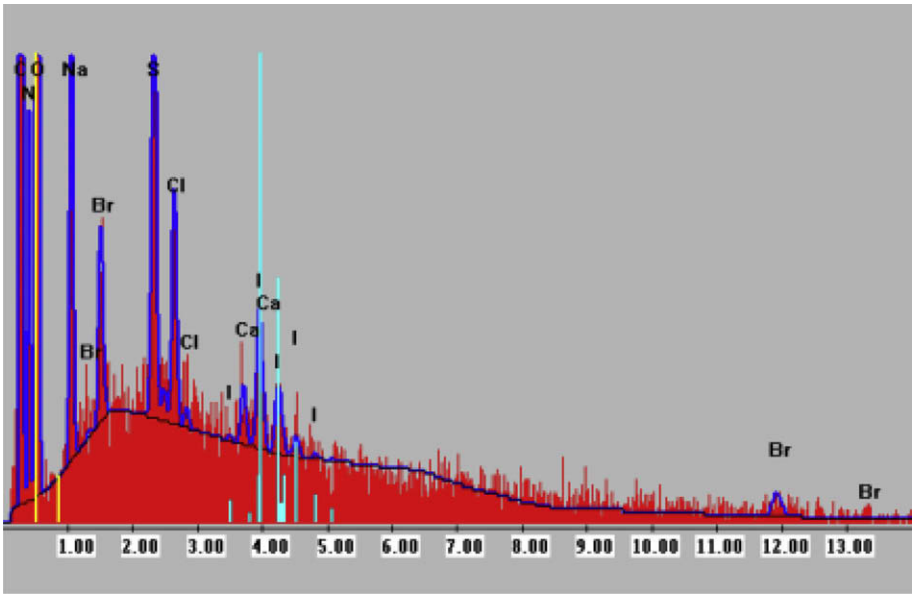

Figure 3 Elemental analysis (EDX/ESEM) of *V. gigantea* fiber.

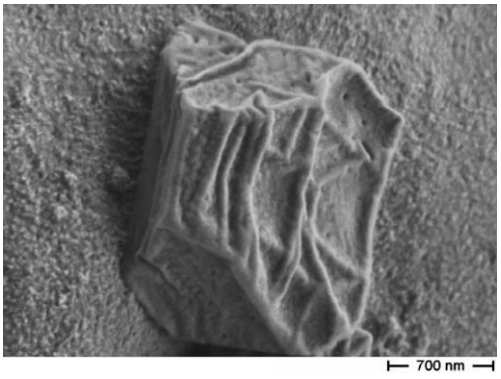

Figure 4 Calcium-based nanoparticle observed in alkaline extracts using SEM.

sponge skeleton. Alkali-resistant crystals were observed by SEM in the related pigment-containing extracts obtained after this kind of demineralization (Fig. 4).

After the demineralization procedure, the skeletal fibers of the marine horn sponge *V. gigantea* as well as isolated crystalline formations (Fig. 4) were investigated by vibrational spectroscopy techniques FTIR and Raman spectroscopy. The recorded FTIR spectra of the demineralized material were compared with the reference α- and ß-chitin spectra. It was found, that the bands in the spectrum of the sample are identical to that of the reference spectrum of α-chitin (Fig. 5), whereas the spectrum of β-chitin exhibits significant differences.

The amide I bands ascribed to the vibrational modes of the CONH group appear in the spectrum of demineralized skeleton material of *V. gigantea* at 1660 cm^{-1} and 1625 cm^{-1}, and the amide II bands appear at 1560 cm^{-1}. Amide III bands can be found in the region between 1315 cm^{-1} and 1200 cm^{-1}. Furthermore, four strong bands appear at 1157 cm^{-1}, 1117 cm^{-1}, 1084 cm^{-1} and 1039 cm^{-1} and can be ascribed to the C–O–C and C–O stretching modes of the protein. The rocking vibration of the methyl group can be found at 1382 cm^{-1}.

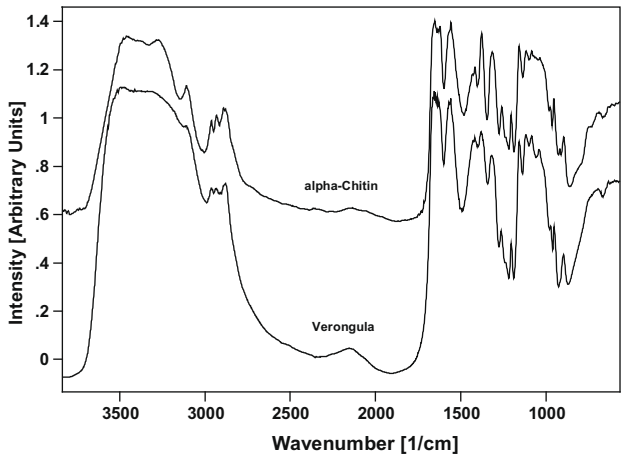

Figure 5 FTIR spectrum of the Verongula sample (bottom) in comparison to the spectrum of the α-chitin reference.

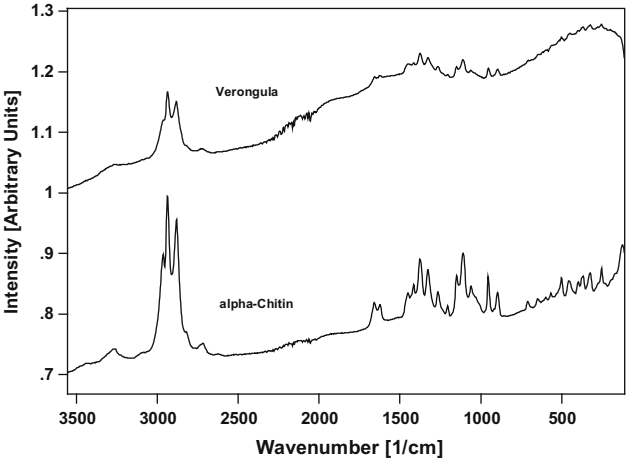

Figure 6 Raman spectrum of the Verongula sample (top), compared to the spectrum of the α-chitin reference.

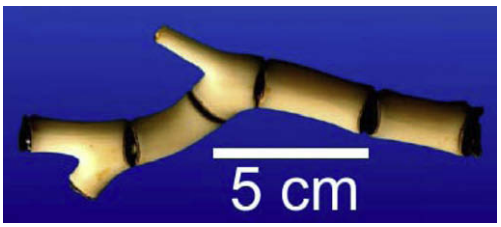

Figure 7 Fragment of a bamboo coral node (Isididae).

All the characteristic bands from the reference spectrum of α-chitin can be found in the FT-Raman spectrum of the demineralized skeleton material of *V. gigantea* (Fig. 6). The spectrum is also in good agreement with the literature results (Galat and Popowicz, 1978; De Gussem et al., 2005). The mineral crystals represented in Fig. 4 were identified using FTIR and Raman spectroscopy as aragonite (data not shown here).

3.1.1. Corals

Bamboo corals are often found at depths of more than 1000 m. These corals have joined branches made of bony calcareous structures alternating with nodes made of a protein-based material called gorgonin. This gives the skeletal structure of the coral an appearance that resembles fingers (Fig. 7).

The skeletal structure and the dimensions of bamboo corals are almost identical to those of bone. We used high sensitivity Raman spectroscopy for the identification of the mineral compound responsible for the mechanical stability of the coral node. The obtained results show the presence of calcite (Fig. 8).

A decalcification procedure by Osteosoft treatment was used to gain understanding of the nature and nanostructure of the coral's axial organic matrix. Step by step demineralization was observed using light microscopy (Fig. 9). Demineralization using Osteosoft solution has led after three days to the appearance of the organic matrix on the surface of the partially demineralized fragment as represented above (Fig. 9).

On the seventh day of the decalcification we observed only the presence of a transparent gelatinous pellicle (Fig. 10) which indicates the complete dissolution of the calcite based axis

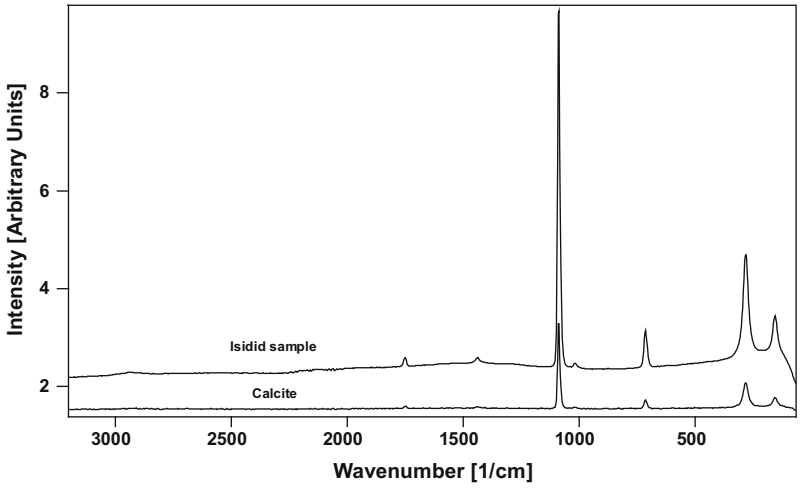

Figure 8 Raman spectrum of *Isidella* sp. coral (top), compared to calcite (bottom).

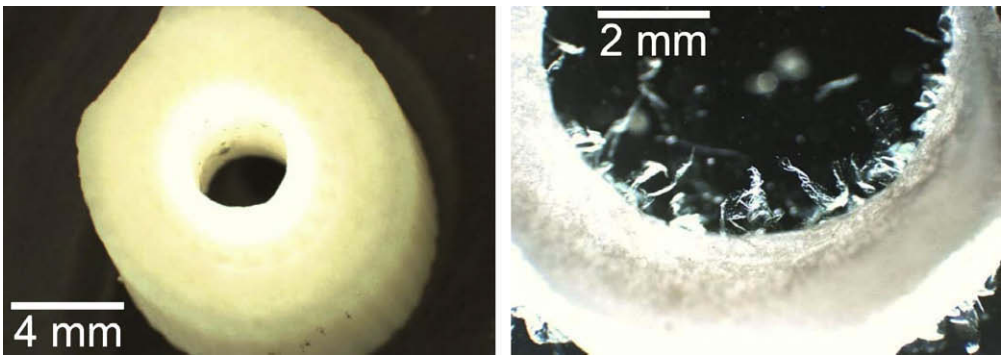

Figure 9 Light microscopy images of fragments of the calcitic internode before demineralization (left) and after partial demineralization (right).

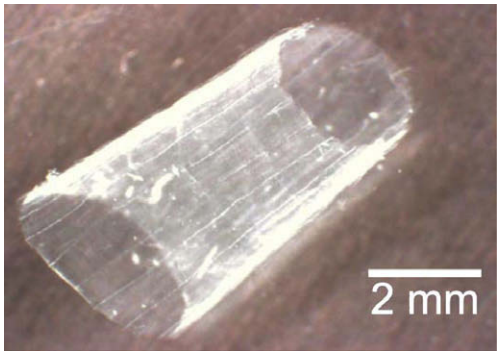

Figure 10 Light microscope image of the organic matrix isolated after the demineralization of the *Isidella* sp. skeletal node.

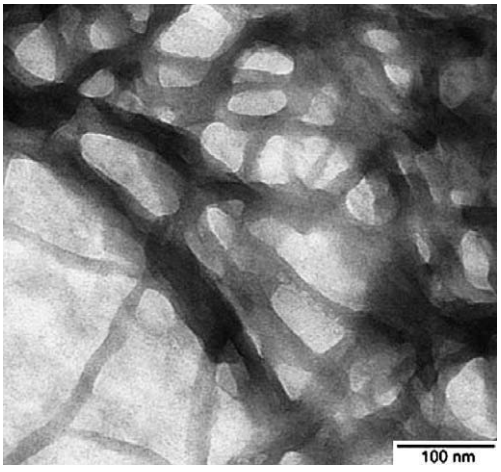

Figure 11 TEM image of the coral organic matrix.

internode. The material forms a continuous central canal through the internode, through the node, and into the adjacent internode. The organic matrix, investigated by transmission electron microscopy (Fig. 11), shows a nanofibrillar protein like structure.

The results of the amino acid analysis of this organic matrix confirmed that glutamine and proline (25.9% and 26.0%, respectively) are the dominant components among the amino acids detected. The very low content of glycine (2.5%) rules out the possibility of the fibrillar matrix being of a collagenous nature. These results are in accordance with previous reports (Ehrlich et al., 2006). Therefore, we conclude that the organic matrix of the Isididae axial internode is an example of an acidic fibrillar protein.

4. Conclusion

Demosponges of the Verongida family including *V. gigantea* have unique potential for use as biomaterial due to the multilayered structure of their rigid fibrous skeletons made of chitin. Results of FTIR and Raman spectroscopy unambiguously showed that the investigated Verongida sponges have α-chitin as the main skeletal component.

The inorganic part within the deep-sea octocoral *Isidella* sp. (Isididae:Gorgonacea) was clearly identified as calcite by Raman spectroscopy. The organic part, isolated by a demineralization process in Osteosoft solution at 37 °C over 10 days, is suggested to be a nanofibrillar network formed from acidic proteins. This kind of nanoorganized organic matrix is suggested to be the template for calcite formation in bamboo corals.

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