

Discovery of a honeycomb structure in the twisted plywood patterns of fibrous biological nanocomposite tissue

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Abstract

Electron microscopy and synchrotron Bragg diffraction were used for the investigation of the structure of the exoskeleton of the lobster *Homarus americanus*. The study reveals a pronounced microstructure hierarchy and a strong crystallographic and topological texture of the α -chitin–protein network underlying the twisted plywood (Bouligand) structure. The results suggest that the classical picture of such structures must be refined. Instead of a smoothly misoriented stacking sequence of its constitutive nanofibrous chitin–protein planes, two major and two minor orientation branches of the fibers perpendicular to a common $\langle 020 \rangle$ crystallographic axis pointing towards the surface of the cuticle were found. This crystallographic texture confirms the microscopical observation that the chitin–protein arrays which form the structural subunits of plywood patterns assume the form of planar honeycombs. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Many fibrous biological nanocomposite tissues based on chitin, collagen, or cellulose reveal a high degree of hierarchical organization (Fig. 1) [1,2].

The most characteristic feature of such materials, visible already at light optical resolution, are mesoscale arch structures which are referred to as Bouligand or twisted plywood patterns (Fig. 2) [3]. According to earlier work [4–7] these structures consist of helicoidal stacks of planar fibrous polysaccharide–protein arrays (Fig. 1). The thickness of one such plywood or Bouligand layer

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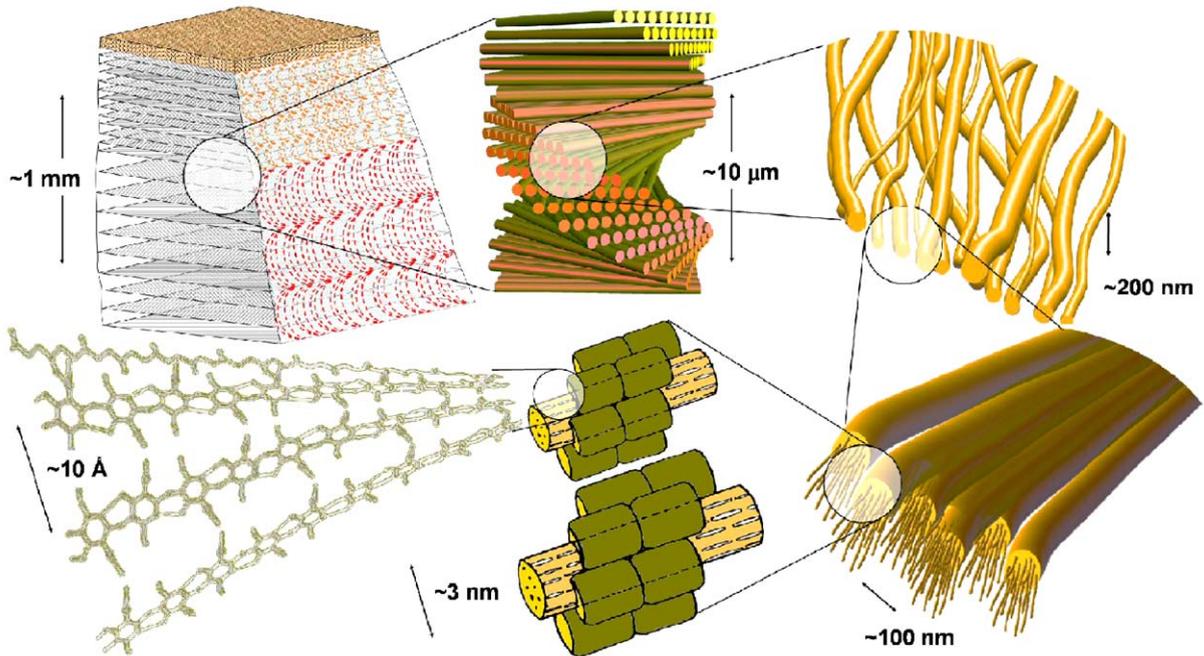


Fig. 1. Structural elements of the exoskeleton material (exocuticle, endocuticle) of *Homarus americanus*. The most characteristic feature of the material is its hierarchical organization which reveals six main different structural levels [1–11]. The first level is the polysaccharide molecule (chitin). The antiparallel alignment of these molecules forms α -chitin crystals [12]. The second structural level is the arrangement of 18–25 polysaccharide molecular chains in the form of narrow and long crystalline units, which are wrapped by proteins, forming nanofibrils of about 2–5 nm diameter and about 300 nm length [13,14]. The third level is the clustering of some of these nanofibrils into long chitin–protein fibers of about 50–300 nm diameter (Fig. 2). The fourth level in the hierarchy is the formation of a planar woven and systematically branched network of such chitin–protein fibers (Fig. 2). The spacing between these strands is filled by a variety of proteins and biominerals (crystalline and amorphous calcite in the case of lobster). The fifth level, visible already in an optical microscope, is usually referred to as a twisted plywood or Bouligand pattern [3–7]. This level is created from the woven chitin–protein planes. Their gradual rotation from one plane to the next creates complex structures (sixth level) which appear as fibril arches when viewed in cross sections (Fig. 2).

corresponds to a certain stacking sequence of planes which are gradually rotated about their normal axis. In this study we show that this generally accepted picture of the inner structure of fibrous biological tissues must be refined. In our experiments on the cuticle of the lobster *Homarus americanus*, we observed in particular that the plywood structure reveals a more complex internal order of its constituent fibers and planes than commonly assumed.

2. Sample material and experimental details

The experiments were conducted on *Homarus americanus*. This is a large arthropod (joint-limb

animal) which belongs to the class of the crustaceans and the order of the decapods. Its cuticle consists, like that of most arthropods, of the three main layers: epicuticle, exocuticle, and endocuticle (Fig. 2). The epicuticle (outer skin) is a very thin and waxy layer which acts as a diffusion barrier to the environment. The exocuticle and endocuticle layers, which carry the mechanical loads, consist of a hard fibrous chitin–protein tissue containing considerable amounts of calcium carbonate minerals (typically crystalline or amorphous calcite) of nanoscopic size [8–11]. These two layers (exocuticle and endocuticle) reveal the well-known Bouligand structure which is characteristic of the arthropod cuticle [3–7].

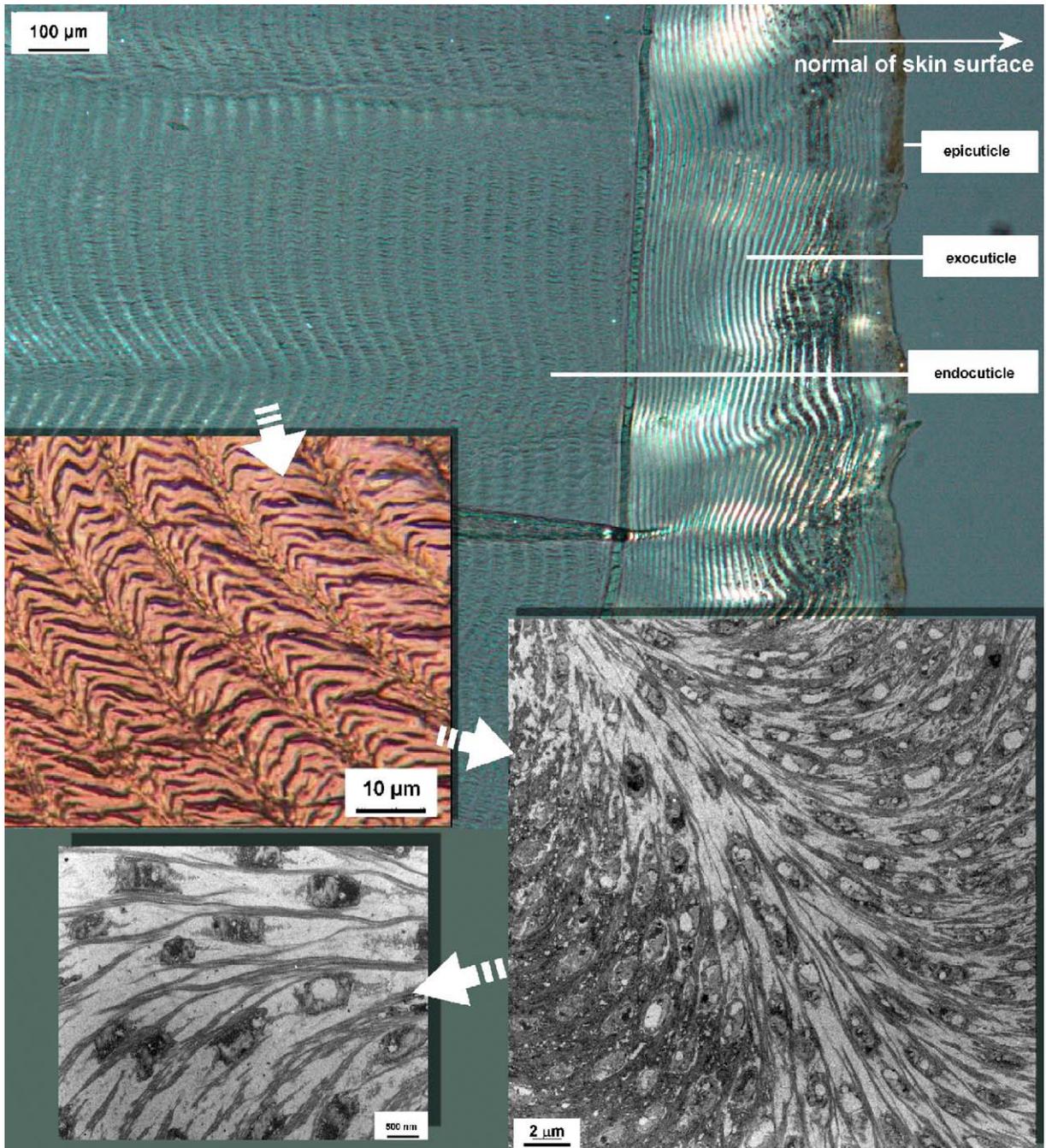


Fig. 2. Microstructure hierarchy in the exoskeleton of the lobster *Homarus americanus*. The images were taken by optical and electron microscopy. The micrographs reveal the hierarchical organization of the material particularly inside the twisted plywood (Bouligand) pattern.

For microstructure characterization, specimens for this study were cut from the dried left cheliped (crusher claw). We employed optical microscopy (Leica DM 4000B), scanning electron microscopy (SEM, CamScan 4), and transmission electron microscopy (TEM, Hitachi H600). Samples for SEM were gold coated. Samples for reflection light optical microscopy were prepared by polishing and gold coating. Samples for transmission light optical microscopy were cut down to 5 μm thickness by using a rotary microtome (Leica RM 2165). Samples for TEM were fixed with 2.5% glutaraldehyde, decalcified with EDTA, stained and fixed with OsO_4 , and finally stained with uranyl acetate and lead citrate to improve contrast. Afterwards, samples were ultramicrotomed down to sections of 60 nm thickness. Some of the specimens were additionally treated with NaOH (5 vol%) to remove the protein structure.

3. Results and discussion

In accordance with earlier studies, Fig. 2 shows that the tissue of the exo- and the endocuticle of *Homarus americanus* reveals two main microstructural scales. At the mesoscale (optical resolution) the matrix shows a twisted plywood (Bouligand) pattern, which is characteristic of the cuticle of most arthropods [3–7]. From our electron micrographs, we can confirm that this Bouligand-type matrix is itself formed by stacks of mutually misoriented chitin–protein fibril planes which contain embedded biominerals (Figs. 1 and 2). The microfibrils in these planes, which appear in dark gray in the electron micrographs, have a diameter of about 50–300 nm. Each of these microfibrils consists itself of a bundle of parallel nanofibrils which contain the actual polysaccharide molecular chains wrapped by proteins (Figs. 1 and 2). These observations are consistent with Refs. [3–7].

A quantitative microscopical study of the inner structure of the Bouligand pattern is difficult though owing to the influence of stereology. This means that the appearance of the patterns depends on the angle at which the sample was cut. Therefore, we decided to apply synchrotron

wide-angle X-ray diffraction for a more detailed study of the arrangement of the nanofibrils within the mesostructure. The advantage of this approach is that the nanofibrils are crystalline, i.e., the polysaccharide molecules arrange in the form of the α -chitin phase which assumes an orthorhombic crystal structure with the lattice parameters $a = 4.74 \pm 0.02 \text{ \AA}$, $b = 18.86 \pm 0.01 \text{ \AA}$, $c = 10.32 \pm 0.04 \text{ \AA}$, and $\alpha = \beta = \gamma = 90^\circ$ [12]. Furthermore, the elongated crystalline arrangement of the molecules within the nanofibrils means that their *crystallographic* orientation distribution reflects also the *topological* orientation distribution of their longitudinal axis. Only when discussing rotations of the crystalline nanofibrils about their common longitudinal axis one has to carefully distinguish between the crystallographic and the topological orientation.

The measurements were carried out at the synchrotron radiation source at HASYLAB/DESY (Hamburg, Germany). We used a monochromatic beam with a wavelength of $\lambda = 0.196 \text{ \AA}$ in wide-angle Bragg diffraction mode. An area detector was used for the measurement of Debye–Scherrer frames from which texture-corrected theta scans for phase analysis and pole figures for texture analysis were obtained. Fig. 3 shows the two pole figures, $\{100\}$ and $\{020\}$, after background correction, normalization, and volume correction. The reference system of coordinates for the projection of the two pole figures is the normal direction of the claw surface (upper direction in the pole figures) and the transverse direction within the cuticle cross section.

Fig. 3a shows that the texture of the chitin–protein nanofibrils is characterized by a very strong fiber texture with a crystallographic $\langle 020 \rangle$ axis parallel to the surface normal axis of the cuticle. This means that the b -axis (*long axis of the lattice cell*) of the α -chitin crystals points toward the surface of the exoskeleton within an orientation spread of about 15° . It must be emphasized that such a strong crystallographic texture is very uncommon even in man-made materials. Such a strong preferred orientation distribution of the nanofibrils could by no means be expected from the micrographs shown in Fig. 2 or published in earlier works [3–7].

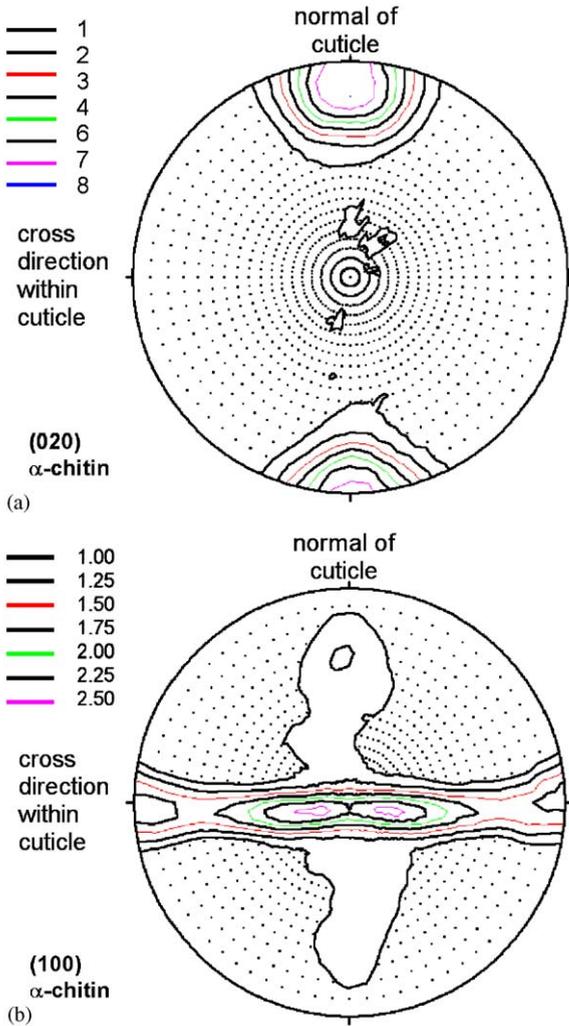


Fig. 3. Pole figures of the α -chitin fiber network within the Bouligand structure measured in the claw of *Homarus americanus* by using synchrotron radiation; 2 mm \times 5 mm sample section; 1 mm \times 1 mm beam size; rectangular sample shape. The reference coordinate system for the two projections are the top (plane surface) and in-cuticle transverse directions of the claw, respectively. The wavelength was $\lambda = 0.196 \text{ \AA}$.

A second important outcome of the orientation data ($\{100\}$ pole figure in Fig. 3b) is that the occupation of the $\langle 020 \rangle$ fiber texture is not isotropic but shows two pronounced maxima of the $\{100\}$ peaks which are misoriented by about $\pm 25\text{--}30^\circ$ and two weaker maxima which are misoriented by about $\pm 85\text{--}90^\circ$ from the pole figure center within the equatorial plane. This

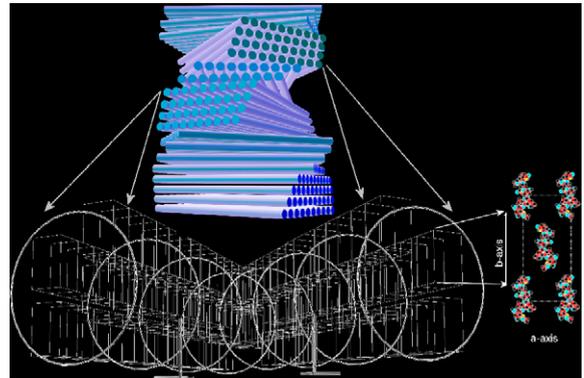


Fig. 4. Schematic drawing of the main texture components of the chitin nanofibrils in the Bouligand structure.

means that the chitin–protein fibers do not only have a common $\langle 020 \rangle$ axis pointing at the cuticle surface but also two major and two smaller preferred crystalline branches within the cross section of the network (Fig. 4). This observation resembles earlier results which were reported by Weiner et al. [15] on preferred topological orientations (30° and 70°) which they found in twisted plywood arrangements of the collagen fibril arrays in primary lamellar bone of rat. When comparing the texture measurements (Fig. 3), particularly the preferred angles close to 30° and 90° in the cross-section plane, with the micrographs shown in Fig. 5, it becomes obvious that the chitin–protein fibers are arranged in the form of a honeycomb structure which is reported here for the first time in the literature.

Following earlier investigations on the nature of the excellent fracture toughness typically reported for fibrous biological nanocomposites [16,17], two main reasons are conceivable for the occurrence of such a pronounced crystallographic and topological texture of the chitin–protein fibers. The first one is the possibility of an enhanced resistance against crack propagation created by such microstructures: one may speculate that an orientational discontinuity in the stacking sequence of the mutually misoriented chitin–protein planes has advantages for crack path deflection or crack branching as opposed to a smooth orientational transition from plane to plane which would be less suited to impede crack propagation. Second, the

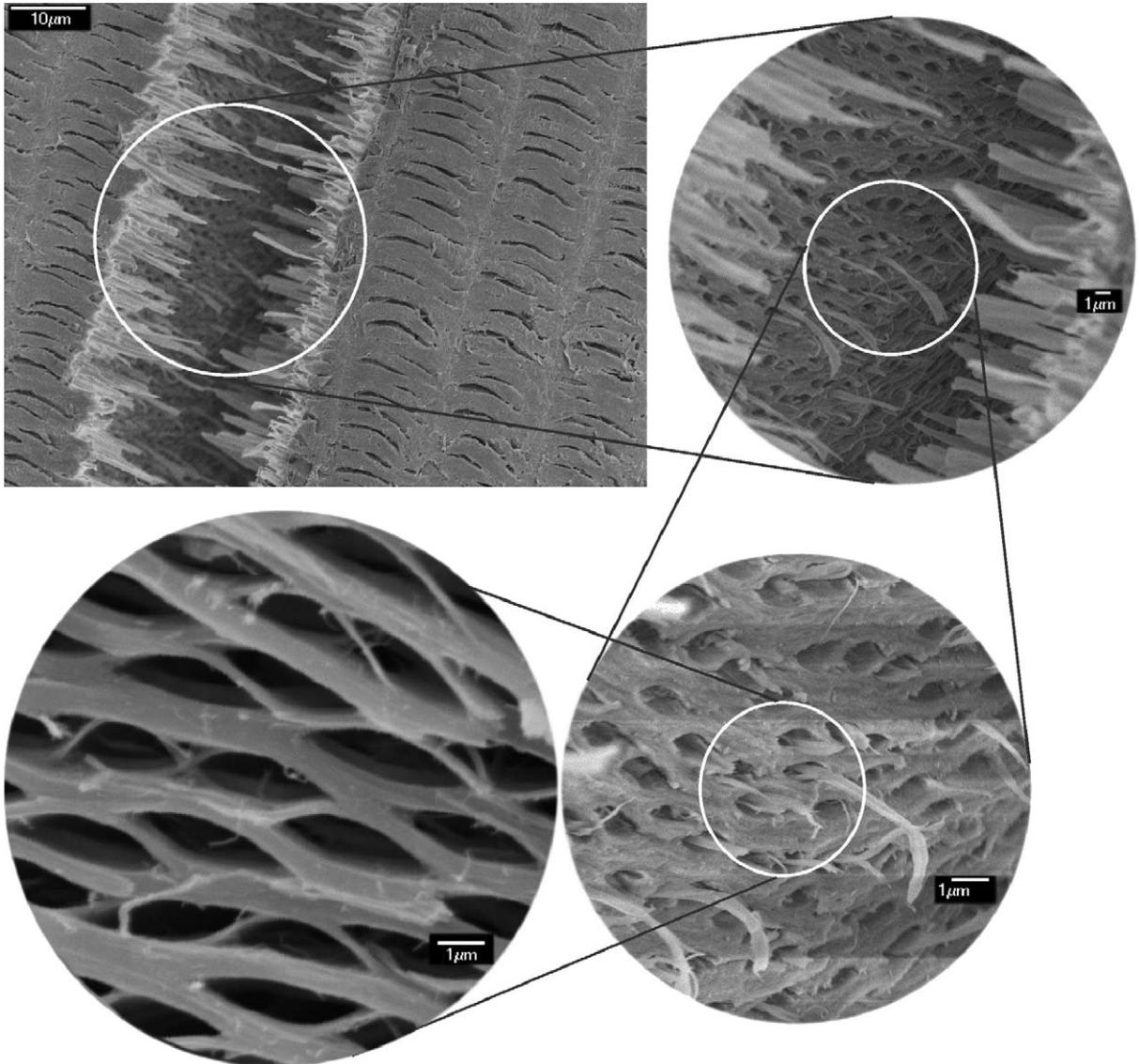


Fig. 5. Some electron micrographs (SEM) taken from fractured specimens of *Homarus americanus*. The images underline the important role of the interwoven inner hierarchy of Bouligand structured material. The observation of the honeycomb-type arrangement of the chitin–protein fibers visible at larger magnifications is consistent with the texture measurements obtained by synchrotron wide-angle Bragg diffraction (Fig. 3).

chitin planes do not simply consist of parallel bundles of nanofibrils but of planar honeycomb-type arrays of fibers which are penetrated by equivalent planar structures under some oblique angle (Fig. 5). This type of arrangement provides a very high density of interfaces

which have to be critically stressed against frictional forces upon fiber pull-out. Such a mesh of two (or more) interpenetrating planar honeycomb-type structures should provide a very high mechanical stability against fracture initiation and propagation.

4. Conclusions

We investigated the internal mesostructure of the twisted plywood structure of the lobster *Homarus americanus*. Particular attention was placed on the structure of the α -chitin–protein planes which create the twisted plywood pattern characteristic of the arthropod cuticle. We found that these planes underlying the twisted plywood structure do not consist of arrays of parallel α -chitin–protein fibers, but form planar honeycombs where the fibers are connected in hexagonal arrays. The observations were discussed in terms of the mechanical stability of such biological composites.

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