Structural and mechanical characterization of chitin using correlative AFM-TEM microscopy and spectroscopy

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Keywords: chitin, protein, AFM, TEM, EELS

Chitin is the most abundant nitrogen-bearing organic compound in nature and the second most abundant natural polymer on earth after cellulose. Chitin is a common constituent of the arthropod exoskeleton in general, including insects, crustaceans, chelicerates, and myriapods. The most characteristic feature of the chitinous cuticle, which is a biological nanocomposite material, is its strict hierarchical organization, which reveals various structural levels: First, at the molecular level is the polysaccharide chitin itself. Its antiparallel alignment forms alpha-chitin crystals. The second structural level is the arrangement of 18-25 of such molecules in the form of narrow and long crystalline units, which are wrapped by proteins, forming nanofibrils of about 2-5 nm in diameter and about 300 nm in length. The third step in the scale consists of clustering of these nanofibrils into long chitin-protein fibrils of about 50-300 nm in diameter. These chitin-protein fibers form a planar woven and periodically branched network (chitin-protein layers). The spacing between the fibers is filled up with proteins and biominerals of microscopic and nanoscopic size [1, 2].

Until now the structural organization of the chitin-protein fibrils has been revealed by various microscopic and analytical techniques with different power of resolution. Usually, X-ray diffraction, TEM, and SEM at high resolution have been used to determine the orientation of fibers, while the protein content of the chitin nanocomposite has been examined by protein extraction followed by two-dimensional gel electrophoresis, immunocytochemistry etc. [2]. Although each of the above mentioned techniques brings a big impact to the understanding of chitin structures, the native multicomponent (polysaccharide chains, proteins, lipids, biominerals) organization of the chitin-based nanocomposite can hardly be observed directly by any of those techniques. The reasons are restrictions in preparative and visualization techniques, e.g. low electron microscopy contrast of proteins, the necessity to use a two-dimensional projection of the sample volume and the issue of beam damage in the case of electron microscopy, the necessity to roughly dehydrate the sample and therefore damage the protein structure for X-ray diffraction and decalcification of the samples.

In this study we propose a correlative AFM-TEM analysis for chitin-protein structural investigations [3]. This method allows making correlative AFM/TEM analysis of the same particular specimen area by two microscopic methods. Here, the crucial point is that one particular organelle or biomolecule can be cut into two parts, one part being used for AFM and another one for TEM and supplemental analytical techniques (EFTEM, EDX, EELS). For AFM, we used the block-face of high pressure frozen and epoxy fixed and embedded cuticle samples of wasp *Cotesia glomerata*, mite *Otodectes cynotis* and *Ligia italica*, while the last ultrathin section was collected, post stained and then used for TEM. Such complementary analysis enables the investigation of:

M.A. Pabst, G. Zellnig (Eds.): MC2009, Vol. 2: Life Sciences,
DOI: 10.3217/978-3-85125-062-6-295, © Verlag der TU Graz 2009

1) the protein content of the chitin chains at the level of nanofibrils (the diameter of thinnest nanofibrils which have been detected by AFM have a diameter of about 3-5 nm which is in good agreement with the theoretical data),
2) the general organization of the chitin-protein nanofibrils network on the surface of the section plane using AFM topographical and phase imaging,
3) the structural organization of the cuticle secreting cell layers using both AFM and TEM,
4) mechanical characteristics of cuticle using AFM phase imaging,
5) chemical composition of cuticle using EFTEM, EDX, EELS.

Hereby the direct imaging of gently preserved chitin-protein cuticles opens up new horizons for the investigation of structural and dynamic processes at the level of individual macromolecular components.

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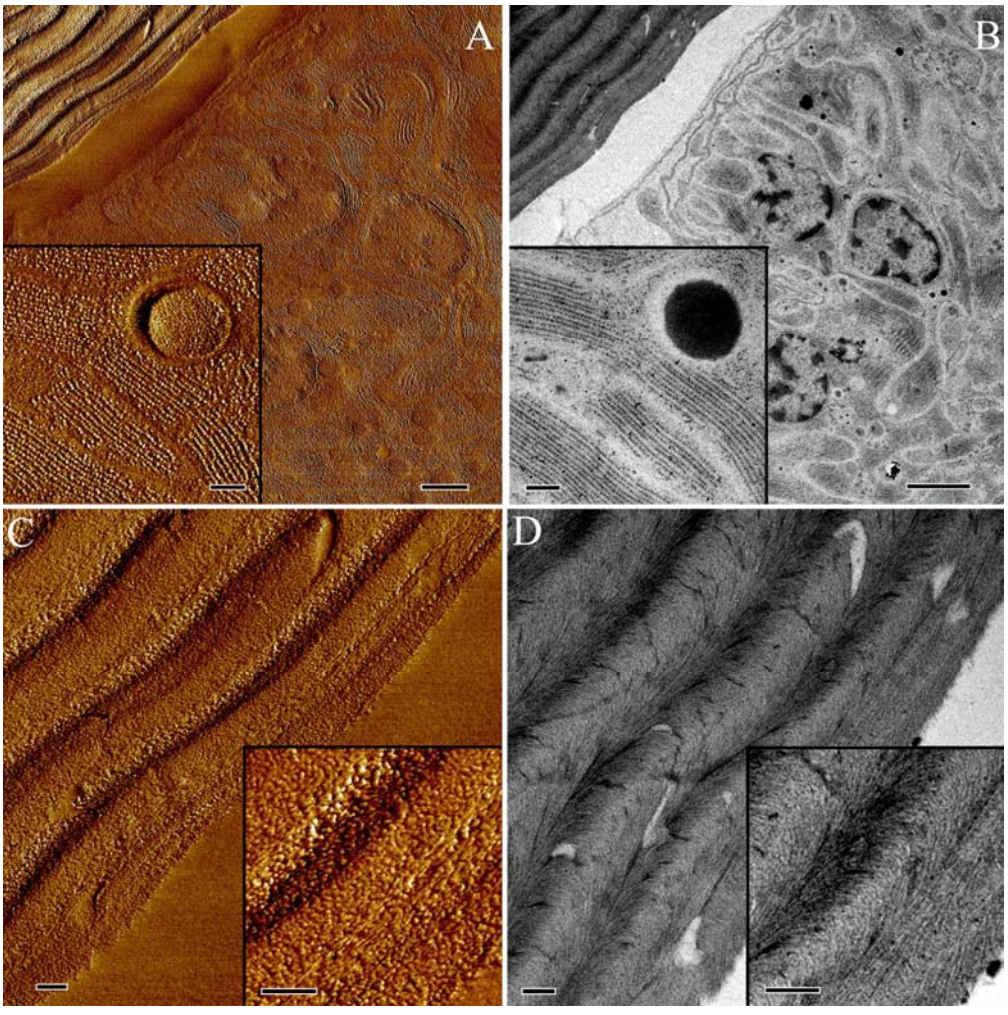

Figure 1. Corresponding (A,C) AFM phase (block face) and (B, D) TEM (ultrathin section) images of mite *Otodectes cynotis*, high-pressure frozen and epoxy freeze-substituted. The insets show elements of structures at high magnification. Phase variations: 0-40° in (A) and 0-25° in (C), scale bars equal 2 μm in (A, B) , and 250 nm in (C, D) and all insets.

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