Chitin

Correlative AFM -TEM Microscopy and Spectroscopy

In this article we present a universal method for structural investigation of chitin by using correlative AFM-TEM microscopy. The advantage of this method is that polysaccharide chains, proteins, and biominerals can be analyzed simultaneously by AFM (protein-chitin fibril distribution) and analytical TEM (chemical composition of the same particular area).

Introduction

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].

Methods

Until now, the structural organization of the native chitin 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. Raman or infrared analysis is the most popular method to determine the mineral composition; while the protein content of the cuticle 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 the chitin structure, the original organization of the native chitin (polysaccharide chains, proteins, and biominerals in their original places within the cuticle) cannot be visualized by any technique until now.

The reason is that each component requires special experimental conditions, and as a consequence, a different measuring technique, which is usually optimal for one and not suitable for the other two chitin constituents. For example demineralization and protein hydrolysis is required in order to investigate the polysaccharide chitin structure by X-ray diffraction. Precise protein analysis is usually done using protein solutions extracted from the rest of the cuticle. For the investigation of the mineral content of chitin by Raman or infrared analysis, the sample has to be dried, so the native hydrated shape of the proteins is completely destroyed.

**Correlative AFM-TEM**

We propose here the universal method for the investigation of the chitin structure in its native like state by using a correlative AFM-TEM analysis. The advantage of this method is that all three components can be preserved simultaneously in the sample using high-pressure freezing/epoxy freeze-substitution methods and then visualized using both (AFM and TEM) high resolution microscopical methods. AFM is applied for the visualization of protein-chitin fibrils, protein-mineral complexities and their mechanical properties, while conventional and analytical TEM (elemental mapping by energy filtering TEM (EFTEM), electron energy loss spectroscopy (EELS) and energy-dispersive X-ray spectroscopy (EDX)) are utilized for the detailed analysis of the chemical composition of the sample. The strongest feature of the AFM-TEM correlative method is that the information can be obtained from the same particular specimen area (same organelle, same chitin-protein fibril etc.). The only requirements are an appropriate sample preparation procedure (fixation, embedding) and optimal AFM and TEM measurement conditions.
Sample Preparation

The ultrastructural appearance can be deliberately influenced with the standard aggressive chemicals like osmium tetroxide which are conventionally used during chemical fixation or freeze-substitution procedures. Such protocols usually partially, or even completely, degrade the cytoplasmic and membrane proteins [3]. Another disadvantage of the OsO4 usage is that osmium containing samples cannot be used for analytical TEM investigations. For AFM-TEM correlative microscopy the optimal sample fixation is high-pressure freezing/epoxy freeze substitution. High pressure freezing provides the best structural preservation of the sample. Epoxy stabilization during freeze-substitution and embedding guarantees the highest quality of AFM images of the cuticle. The usage of hydrophilic resins (e.g., most acrilates and methacrilate based resins) which are spread widely because of their high suitability for "on-grid" immunolocalization do not provide a sufficient structural contrast, neither for cytoplasmic components nor for chitin ultrastructure (fig. 2).

Results

It appears to be clear that a comprehensive understanding of the role of proteins in the native chitin can be obtained not only by investigation of the extracted proteins, but also by structural analysis of the distribution of the protein matrix within the cuticle which is preserved as accurately as possible. Figure 3 represents the complementary couple of images of the cuticle of the mite. The sample has been high pressure frozen/epoxy freeze substituted and epoxy embedded. As it has been mentioned above, this method provides the most accurate (after high pressure freezing/cryo sectioning) protein preservation of biological materials [4]. The ultrastructure of the cuticle is manifested differently in TEM and AFM images. The TEM image shows only those polysaccharide-protein fibers which can be stained properly and which can be visualized as separate constituents of the sample volume in a 2D projection of the 50 nm thick section layer (TEM projection issue). The AFM phase image contains both: The oriented polysaccharide fibers (nicely oriented patterns of thin filaments) and the protein macro-molecules, which appear as grained structures around and between the chitin fibers [4]. Also the phase image depicts the different stiffness of the proteins layers within the cuticle: Each two neighboring layers contain the soft area (dark phase contrast) in between (fig. 3). As the mite cuticles do not contain minerals in a concentration which can significantly influence the stiffness of layers (EDX spectra obtained from regions one and two (fig. 3) show almost the same chemical content), one can suggest that the different stiffness can be attributed mostly to the different protein composition of these regions.
It has to be mentioned that the protein distribution within the biological chitin is also not homogeneous at larger scales (micrometers). This effect can be detected perfectly by AFM phase images with very high spatial resolution (fig. 4). TEM micrographs of the longitudinal section of mite cuticles indicate quite uniform fibril thicknesses, as well as their orientation patterns. In contrast to the TEM image, AFM phase images show that the chitin-protein fibrils have a great variety of orientation patterns and also an essential difference in the size distribution. Because of the projection issue and low contrast for light elements (C, O, H), such information is usually missing in TEM data.

**Biominerals**

Inorganic minerals are used in many biological systems for different purposes, with teeth, bones and shells being only the most prominent examples. Usually in biological composites the mineral precipitates *in situ* swollen polymer matrices within. In the last decade the attention has increasingly turned to amorphous phases that earlier remained mostly undetected due to a lack of suitable analytical techniques. Crustaceans are excellent models to investigate Biomineralization. *Ligia Italica* has a rigid cuticle which contains an organic matrix and larger amounts of CaCO$_3$ as the main mineral component. During growth, the cuticle has to be replaced by a new one in a process called moulting, in which a new cuticular matrix has to be synthesized before, and mineralized after the old cuticle is shed [5]. As most of the biological processes, the mineralization is also a highly dynamical process, which makes the structural investigation of the cuticle especially challenging. In order to gain insight into this process, the structural investigation has to be performed very close to the *in vivo* state with a lateral resolution in the range of nanometers. Correlative AFM and TEM analyses including supplementary analytical techniques allow one to successfully localize and indentify both: (I) the mature calcium carbonate deposits and (II) the mineral migration inside the newly mineralized cuticle. Fig. 5 represents an analytical study of the mineral clusters that are localized in the exocuticle area of the mature cuticle.

**Conclusions**

In this particular case, the usage of each of the applied methods (AFM, TEM, EELS, EFTEM, EDX) was necessary in order to give a comprehensive description of the sample. TEM provided information about the general organization of the chitin-mineral distribution in the cuticle, Ca, O, Si have been localized by EFTEM elemental mapping and EDX in STEM mode. Detection of the N by EDX especially in the presence of C and O within the analyzed area is complicated because of peak
overlaps and therefore requires EELS. Summarizing, the correlative AFM-TEM microscopy and spectroscopy approach provides a great potential for the investigation of the native chitin structure on the level of separate nanofibrils.

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References:

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