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Application Notes

Life Sciences

THE SPM DESIGN AND METHODES FOR BIOLOGICAL APPLICATION

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Introduction

Collagen is the most well-known natural polymer and main component of connective tissues of all types. Collagen is a fibril protein. It has filamentary structure.

According to one of well developed concepts of skin sensitivity the collagen geometrical parameters changing during temperature variation render direct influence on pulse activity of the nervous terminations in skin. It appears to be the base of phenomenon of skin sense code for heat and cold formation.

The investigation of structure of collagen is an interesting problem for biologists. It is impossible to use optical microscope for these purposes because of its low resolution. Electronic microscopy gives morphological features of molecules with sufficient resolution but does not allow authentically defining of lateral sizes of these features and estimating surface roughness. Besides that it takes too much time, reactants and vacuum conditions for sample preparation and measurements by electronic microscopy. Biological samples must be fixed, dried and coved with a thin metal film. Such one-stage sample preparation excludes opportunity of dynamic supervision of sample. Atomic Force Microscopy (AFM) doesn't requires complex sample preparation and allow investigating "alive" biological sample under different conditions with nanometer resolutions. The most elementary component of collagen fibrils is a tropocollagen. It's a triplex right-oriented spiral containing three left-oriented polypeptide chains. Amino acid composition of every chain is very abundant by Glycine, Proline, hydroxi-Proline (-Gly-Pro-hPro- - triplets). Areas which are rich with such triplets are proposed to have crystal-like properties and these zones are combined with amorphous areas which are saturated with heavy charged amino acid residues. Three such polypeptide chains are integrated into the tropocollagen by means of non-covalent binds. Tropocollagens are integrated into more and more complex fibrils thanks to the covalent binds. So we can see a thick bundle as the result of this process.

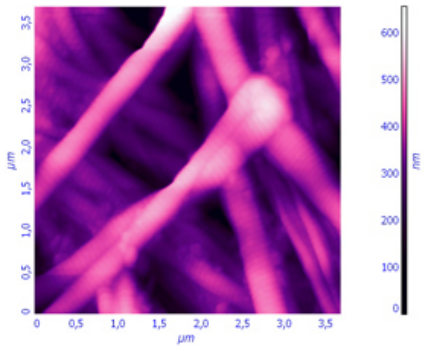


Fig. 1 The thick bundle of collagen image obtained in air by atomic force microscopy (Solver Bio, NT-MDT Co.). The strip periodicities of fibrils can be seen well.

According to the latest structural investigations it has been recently proposed that peripheral sub fibrils have more covalent binds than central and so collagen fibril has a rather hard shell and softer core. It's very probably that mutual hiding tropocollagens terms causes the specific appearance strips which are very clearly observed by AFM methods. The stripes periodicity is 67 nm. However the absolute most of AFM-images were produced on the basis of dried collagen samples. At the same time it's well known that protein conformation is very sensitive to the level of hydratation. We suppose that collagen structure is to be especially sensitive to water regime because 100 g of collagen is able to fix 180 g of water.

The objectives

Detect the morphological parameters changes of the collagen molecules depending on water regime using AFM and to investigate the collagen internal structure using more "hard" AFM scanning mode.

Samples preparations

The collagen of rat tail tendon stretched on glass surface was used. The collagen fibrils have a bed adhesion to substrate surface in water. Collagen was fixed on glass processed with 0,1 % alcyan blue solution for effective adhesion of collagen fibrils. The alcyan blue processing gave a positive charge to the substrate.

Method of investigation

The collagen molecules morphology was investigated by SPM Solver Bio NT-MDT, Russia.

Investigations of fixing sample on air by AFM. The semicontact mode for investigation of the dried fibrils on air was chosen due to the fact that in contact mode we observed the catching of biological sample by SPM-probe. The scan rate was selected at the level of 1-1,5 line/sec.
We used: noncontact Si probe NSG11 (NT-MDT) with spring constant 5 N/m, curvature radius of tip 10 nm, aspect ratio 3:1, noncontact "whisker " type Si probe NSC05 (NT-MDT) with spring constant 11 N/m, curvature radius of tip 10 nm, aspect ratio 10:1.
Investigation of collagen fibrils in liquid by AFM was carried out on glass in a drop of distilled water. The semicontact mode with more "soft" probes for investigation of the fibrils in water was chosen. Scan rate in this method was about 0.5 line/sec. Increase of the scan rate led to stripping of sample from glass surface.
We used: contact Si probe CSG11 (NT-MDT) with spring constant 0,03 N/m and curvature radius of tip about 30 nm. Resonant frequency was selected within15-40 KHz.
Special preparation of the probes for investigation in liquid. Contact Si probe CSG11 was preliminary rebated by scanning the Si sample. The cantilever deflection value was 3-5 times more than during researches with standard contact mode (3-5nA).

Results of investigations

During the investigations of dry sample on air we found that strip periodicity of collagen fibrils was about 67 nm (Fig.2). It absolutely confirmed literature data.

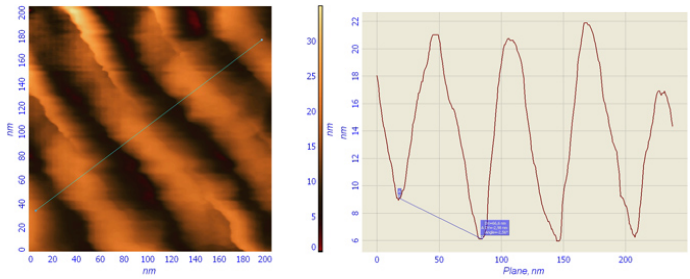


Fig. 2 Topography and surface profile of dry collagen fibril on glass obtained in semicontact mode with noncontact Si probes NSG 11**.** The stripes periodicity of fibril resolved well.

During the scanning of fibril in water we found that stripes periodicity of hydrated collagen was about 54-57 nm. We also observed delicate structure of stripes - each stripe had two additional peaks. It was seen well on fibril profile (Fig. 3). It could be explain both a watering effect and revealing the internal structure of fibril as e result of big pressure on soft and hydrated molecule by AFM-probe.

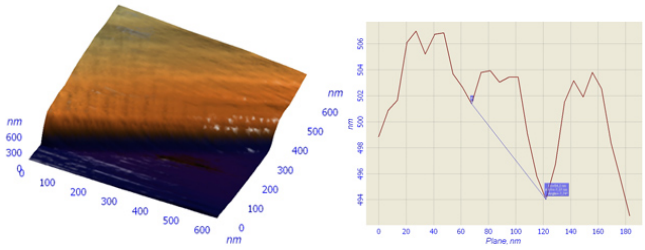


Fig. 3 The collagen fibril in water on glass obtained in semicontact mode with contact Si probe CSG 11. The stripe periodicity of fibril and delicate structure of stripes were observed in surface profile.

During the investigations in water we observed the process of extreme deformation of collagen fibril by AFM tip - the smearing of peripheral layers of the fibrils along glass and displacement of more "hard" core of the fibrils in a quick scanning direction (arrow at fig.4). The periodicity has remained 57 nm. We suppose that it's a powerful argument for the Gutsmann's conception of two-ply structure of collagen fibrils. However we concluded that the central part of fibril is more solid than peripheral part. Our results can be confirmed by X-ray diffraction results of DJ Hulmes et al. They supposed that collagen fibrils resemble smectic, liquid crystals in being highly ordered axially but relatively disordered laterally.

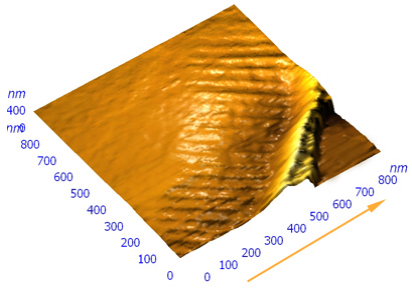
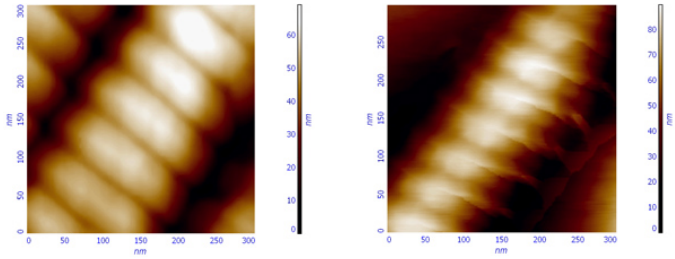


Fig. 4 The collagen fibril in water on glass obtained in semicontact mode with contact Si probes CSG 11. The stripe periodicity of fibril and delicate structure of stripes are observed in surface profile.

Use of the "whisker" type probes. For the best morphological resolution of dried collagen fibrils we used "whisker" type probes (Fig. 5).

The duplication of peaks was not observed. It is possibly caused by changes of glycosaminoglycan surround of fibrils because glycosaminoglycans (highly sulfated polysaccharides) are covalently linked with collagen and can mask the delicate structure of fibrils by environment-dependent manner.



a) b)

Fig. 5 The topography of dry collagen fibril on glass obtained in semicontact mode: a) with noncontact Si probes NSG 11, aspect ratio 3:1.; b) noncontact Si "whisker " type probe NSC05 (NT-MDT), aspect ratio 10:1. It is obvious, that increase of probe aspect ratio leads to essentially increase of morphological features resolution.

Conclusion

The morphological features of collagen fibrils on air and in liquid were investigated. The stripe periodicity on dry fibril is 67 nm and in water is 54-57 nm that could be explained by complex space transformations of tropocallagen orientation caused by water saturation and swelling.
The collagen fibrils have two-ply structure – the central part of fibril is more solid than peripheral part. It was determined by X-ray diffraction earlier. AFM measurements with more "hard scanning" confirmed this fact.
It is possible to assert, that scanning probe microscope allows to investigate not only surface morphology of biopolymers such as collagen, but also "to probe" their internal structure.

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