Ultrastructural Studies on the Collagen of the Marine Sponge *Chondrosia reniformis* Nardo

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The ultrastructure of isolated fibrils of *Chondrosia reniformis* sponge collagen was investigated by collecting characteristic data, such as fibril thickness, width, D-band periodicity, and height modulation, using atomic force microscopy (AFM) and transmission electron microscopy (TEM). Therefore an adapted pre-processing of the insoluble collagen into homogeneous suspensions using neutral buffer solutions was essential, and several purification steps have been developed. Fourier transform infrared reflection−absorption spectroscopy (FT-IRAS) of the purified sponge collagen showed remarkable analogy of peak positions and intensities with the spectra of fibrillar calf skin type I collagen, despite the diverse phylogenetic and evolutionary origin. The sponge collagen's morphology is compared with that of other fibrillar collagens, and the typical banding of the separated single fibrils is discussed by comparison of topographical data obtained using AFM and corresponding TEM investigations using common staining methods. As the TEM images of the negatively stained fibrils showed alternating dark and light bands, AFM revealed a characteristic periodicity of protrusions (overlap zones) followed by two equal interband regions (gap zones). AFM and TEM results were correlated and multiperiodicity in *Chondrosia* collagen's banding is demonstrated. The periodic dark bands observed in TEM images correspond directly to the periodic protrusions seen by AFM. As a result, we provide an improved, updated model of the collagen's structure and organization.

Introduction

Collagen is a common structural protein, occurring in many living systems. It is the major component of the extracellular matrices of all metazoan life, e.g., in glass and horny sponges, bone or tendon of vertebrates, or invertebrate connective tissues.[1−3] The evolution of collagens can be considered as a hallmark of metazoans.[4] From the first multicellular animals (the glass sponges) to humans, only two collagen types appear to have been conserved, i.e., the fibrillar and the basement membrane collagens.[4,5] Sponges are anatomically simple, with a body surrounded by a layer of pinacoderm cells and internal tissue, the mesohyl, consisting of cells and a structured matrix rich in collagen fibrils.[6] In contrast to horny sponges (Demospongiae), where the silica spicules are "glued together" by "collagenous cement" made of microfibrils,[7] fibrillar collagen is also present within the spicules of the older glass sponges (Hexactinellidae) and acts as a template for the biosilification process.[8−10] The fact that fibrillar collagen is present in glass sponges, which date back to the Cambrian (600 million years ago), highlights collagen's fundamental role in the evolution of the earliest metazoan and their skeletons.[11] It can be deduced that living organisms at the earliest stages of metazoan evolution were the first to form highly structured silica networks controlled by collagen; the same protein was later involved in bone and tooth formation.[12]

Collagen is a system which shows a large degree of polymorphism, because its molecules may form a variety of different structures.[13] In addition to the commonest type I, in humans 27 different collagenous polypeptides of manifold ultrastructure are known, which have different and sometimes unknown functions.[14] Each type is made up of triple-stranded subunits, the so-called tropocollagen. These molecules consist of identical (homotrimer) or genetically diverse (heterotrimer) alpha-chains. All alpha-chains contain at least one collagenous domain consisting of a repeating Gly-X-Y triplet, in which X and Y are often the amino acids proline and hydroxyproline, respectively.[15] In the case of fibrillar collagens, specific conditions induce the lateral arrangement of the collagen molecules which leads to self-assembly of right-handed superhelices, fibrils, sometimes showing alternating regions of diverse density (D-period) along the longitudinal axis.[15]

Owing to its low immunogenicity, natural collagen today is applied in medicine, surgery, and cosmetics, e.g., as shields, injectable dispersions, sponges, and microparticles.[16,17] For these purposes, generally acid-supported isolation of the collagen from calf skin is performed.[18] However, bovine collagen may elicit antigenic responses and varies from batch to batch.[19] Reconsideration of bovine collagen as a main source arose with the risks of BSE (bovine spongiform encephalopathy) and TSE (transmissible spongiform encephalopathy).[20] Excepting porcine collagen for example, maybe alternatives can be found in sponge collagens. Until now, only few investigations have yielded information on the ultrastructure and biochemical properties of sponge collagens. Often the characteristic insolubility and mineralization causes methodological problems when isolating and studying the collagen from the collected sponges.[8,21]

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Because of these limitations Swatschek et al. suggested the marine demosponge *Chondrosia reniformis* Nardo (Demospongiae: Hadromerida: Chondrosiidae)[22] as a possible candidate and developed an applicable procedure for standardized collagen isolation.[20] Their study also demonstrated that conventional collagen can be substituted by this marine collagen. Recent interest arose with the application of *Chondrosia* collagen as an organic template for silicification *in vitro*.[23] It has been recognized that the mechanical properties of the biomimetically inspired hybrid xerogels can be improved remarkably due to the presence of the collagen.

TEM, SEM, X-ray diffraction, and electron holography have provided substantial information on collagen ultrastructure and organization.[2,24−26] However, the data obtained using different electron microscopic techniques alone does not usually explain all structural features. Investigations regarding the fine structure and physicochemical properties of the collagen of the marine sponge *Chondrosia reniformis* Nardo[22] started with the studies by Garrone et al.[27] The biochemical analysis of isolated fibrils yielded an amino acid composition similar to that of vertebrate collagen. The infrared spectra obtained from the whole cortex of the sponge showed variations of some typical peaks compared to pure collagen. X-ray investigation data indicated the classical helical structure of *Chondrosia* collagen. An apparent period of about 22 nm and a diameter of about 20 nm were determined. Transmission electron microscopy of stained fibrils exposed a periodic banding pattern of one dark segment alternating with two light segments. It would be desirable to obtain additional information regarding the topography of these segments. The most popular method to gain such information is atomic force microscopy (AFM). AFM is a nonoptical imaging tool, based on the measurement of interaction forces between a sharp silicon or silicon nitride tip and the sample's surface. Because AFM requires comparatively minimal sample preparation, it is suitable for revealing the height distribution of biological samples adsorbed on smooth surfaces.[28,29] Operating in tapping mode means intermittent contact between tip and sample which allows topographical data to be gathered without manipulating the sample.[30,13] Hitherto, the collagen of *Chondrosia reniformis* has not been studied using AFM.

In this work we study the collagen of the marine sponge *Chondrosia reniformis* Nardo in more detail, to obtain continuous information on its ultrastructure. After developing and evaluating a proper purification procedure, for the first time we performed atomic force microscopy to realize in situ investigations of the fibril's organization and surface structure as well as established transmission electron microscopy. Combining the results of both techniques, we can provide an advanced model of the collagen's ultrastructure and organization.

Materials and Methods

1. Isolation and Solubilization of Sponge Collagen. The sponge *Chondrosia reniformis* Nardo was harvested from the Aegean Sea and stored in 50% ethanol until used. The method for isolation of the collagen from the sponge material has been described previously.[20] In brief, the sponge material was washed in water and homogenized in a buffer solution (pH 9.5) using a blender. After 24 h the viscous extract was centrifuged (5000*g*) and the pellet was discarded. The collagen was precipitated from the supernatant by adjusting the pH to 4. After centrifugation (20000*g*) the pellet (collagen) was resuspended in distilled water and frozen until usage. Solubilization experiments were carried out at 4 °C by vigorous stirring of the collagen in various media, e.g., 0.001−1 M hydrochloric acid (Fisher Scientific), acetic acid (Sigma) and 0.01−1 M Tris-based buffer solutions (pH 5.5−9.5, pH was adjusted by adding 5 M HCl), 0.001−1 M sodium hydroxide (Fluka).

2. Purification and Preparation of Homogeneous *Chondrosia* Collagen Suspensions. Fractions (prepared and frozen according to point 1 in this section) of approximately 10 g collagen (about 30% water content) were dissolved under vigorous stirring in 100 mL of 0.1 M Tris/HCl buffer solution (pH 7.4) at 4 °C for 2 days. Trizma base ($NH_2C(CH_2OH)_3$) purchased from Sigma was used for the preparation of the Tris/ HCl buffer. After filtration with mesh sizes down to 200 μm (Mini Sieve Micro Sieve, Bel-Art Products) to remove extant insoluble ingredients (dense parts of the sponge skeleton), the suspension was freeze-dried using a Christ Alpha 1−4 instrument. The lyophilizate (5 mg/mL) was resuspended in 0.1 M Tris/HCl buffer (pH 7.4) and dialyzed (MWCO 12−14 kDa, Roth) at 4 °C against the same buffer solution for 7 days. After another lyophilization, the purified collagen was stored at 4 °C until usage.

3. Preparation of Fibrillar Calf Skin Collagen Type I and Homogenization. Bovine tropocollagen type I lyophilizate (Sigma) was dissolved (1 mg/mL) in 0.01 M acetic acid at 4 °C for 24 h. After centrifugation (10000*g*, 30 min, 4 °C), the supernatant was adjusted to the final concentration. Mixing equal volumes of tropocollagen solution and two-fold concentrated fibrillogenesis buffer at 37 °C led to the formation of fibrils.[31] After 24 h of fibrillogenesis, the suspension was centrifuged. Finally, the supernatant was discarded and the pellet was freeze-dried. Homogeneous suspensions were prepared by stirring of the fibrillar collagen lyophilizate for at least 24 h at 4 °C in deionized water or 0.1 M Tris/HCl buffer solution (pH 7.4).

4. Fourier Transform Infrared Reflection−Absorption Spectroscopy (FT-IRAS). Homogeneous suspensions of the dialyzed (against deionized water) *Chondrosia* collagen or fibrillar collagen type I (homogenized in deionized water) were air-dried on polished titanium discs. Measurements were taken using a Perkin-Elmer spectrometer Spectrum 2000 equipped with an AutoImage microscope.

5. Transmission Electron Microscopy. Negative and positive staining of the collagen fibrils was performed using a slightly modified protocol of Trotter and Koob.[26] After adsorption on the TEM grid (Formvar carbon-coated copper grids, 200 mesh), the fibrils were treated with sodium chloride and fixing solution (glutaraldehyde, sodium acetate, magnesium chloride). Positive staining used tungstate and uranyl acetate, and negative staining used phosphotungstic acid. An acceleration voltage of 120 kV in ultrahigh vacuum was applied to visualize the collagen fibrils with a Zeiss EM 912 transmission electron microscope.

6. Atomic Force Microscopy. Sponge collagen (1 mg/mL) was suspended in 0.1 M acetic acid (pH 4) or 0.1 M Tris/HCl buffer (pH 7.4) or 0.001 M NaOH (pH 12) under constant stirring at 4 °C. After 7 days of suspending, 50 μL droplets of the suspensions were set on the surface of titanium-sputtered glass discs and the collagenous material was allowed to adsorb for 30 min. Finally, the samples were rinsed with deionized water and air-dried. All AFM imaging was performed in tapping mode using a Nanoscope IIIa Bioscope (Digital Instruments/ Veeco) and aluminum reflex-coated silicon tips (force constant 40 N/m). Both deflection and height images were captured simultaneously at imaging speeds of 1.2 Hz, scanning 512 lines. Measurements were taken from height images assisted by WSxM software (Nanotec Electronica).

Results and Discussion

Most of the cortex of *Chondrosia reniformis* appears to be composed of interlacing collagen fibers. Cross-section analysis by Garrone et al. via TEM showed that minute filaments facilitate the connection of single fibrils to build up these intertwined bundles.[27] As the *Chondrosia* collagen isolated by us[20] occurred as such thick and long bundles, our first intention was to check if the nature and the pH of the applied media show a distinct effect on the behavior of the originally irregular collagen fibers.

For fibrils of soluble collagen type I, acid treatment facilitates the degradation into the monomer tropocollagen. Conversely, neutral buffers generally induce the self-assembly of the

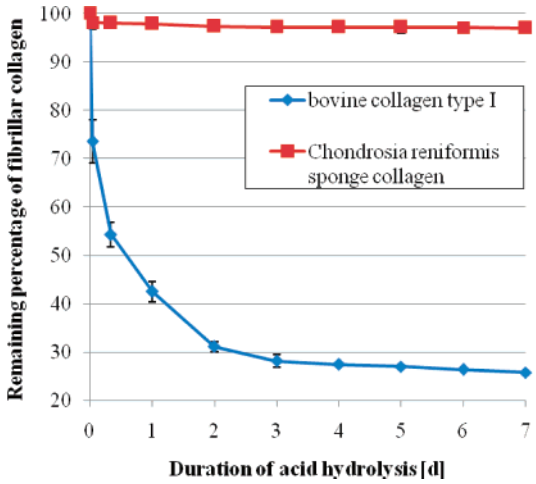

Figure 1. Comparison of the time-dependent change in the percentage of the fibrillar fraction for both bovine collagen type I and *Chondrosia* collagen when treated in 0.1 M HCl at 4 °C.

tropocollagen into fibrils. The graph in Figure 1 compares the time-dependent change in the percentage of fibrillar collagen for both bovine collagen type I and *Chondrosia* collagen. The percentage of fibrillar collagen was measured using the Lowry protein assay as described by Douglas et al.[32] For collagen type I, the chart displays the successive decomposition of the fibrillar content into tropocollagen due to the acid conditions (0.1 M HCl at 4 °C). After 2 days only about 30% of the initial amount remains fibrillar, indicated by hardly soluble or insoluble constituents, obviously originating from the tropocollagen used. In contrast, acid treatment of the purified *Chondrosia* collagen has nearly no effect on the fibrillar content. This result confirms the observations by Imhoff et al.[21] who tried to dissolve the native collagen of *Chondrosia reniformis* to the level of tropocollagen in aqueous solutions. Maybe this fact can be ascribed to strong intrafibrillar cross-links similar to the covalent cross-links that stabilize the fibrillar aggregates in vertebrates; however, for sponge collagen the chemistry of these cross-links is still unknown.[33]

After suspension of the isolated *Chondrosia reniformis* sponge collagen for 7 days in acidic, neutral buffered, or basic media, we visualized the behavior using atomic force microscopy. Even after lengthy and vigorous stirring of this material in acetic acid, almost no separation of fibers from the bundles was observed macroscopically. AFM imaging confirmed this state by showing that the collagen fibrils remain unchanged and arranged in irregularly thick fibers (Figure 2a).

By contrast, using neutral buffer solutions like 0.1 M Tris/HCl (pH 7.4) facilitated formation of homogeneous milk-like suspensions within a few hours of stirring. Even highly concentrated suspensions up to 100 mg/mL were stable for weeks this way. In that case after 7 days the solvent accomplished the plain separation of the fibers into single collagen fibrils as shown in Figure 2b. The fibrils are remarkably uniform and mostly arranged side by side. Additionally they appear to be quite flexible and exhibit no breaks or abrupt kinks as often seen for collagen type I. It is assumed that the solvent unstitches the already described interfibrillar filaments, which are believed to be responsible for the aggregation of the fibrils to form fibers.[27] Because the length of observed single fibrils, measured by coupling wide-range AFM images, amounts up to several hundred micrometers and taking into account reported and measured average diameters of about 20 nm, this results in remarkable aspect ratios of about 1:5000 and more. This correlates to previously reported data on other invertebrate collagen fibrils (spine ligament of *Eucidaris tribuloides* 1:2500,[34]

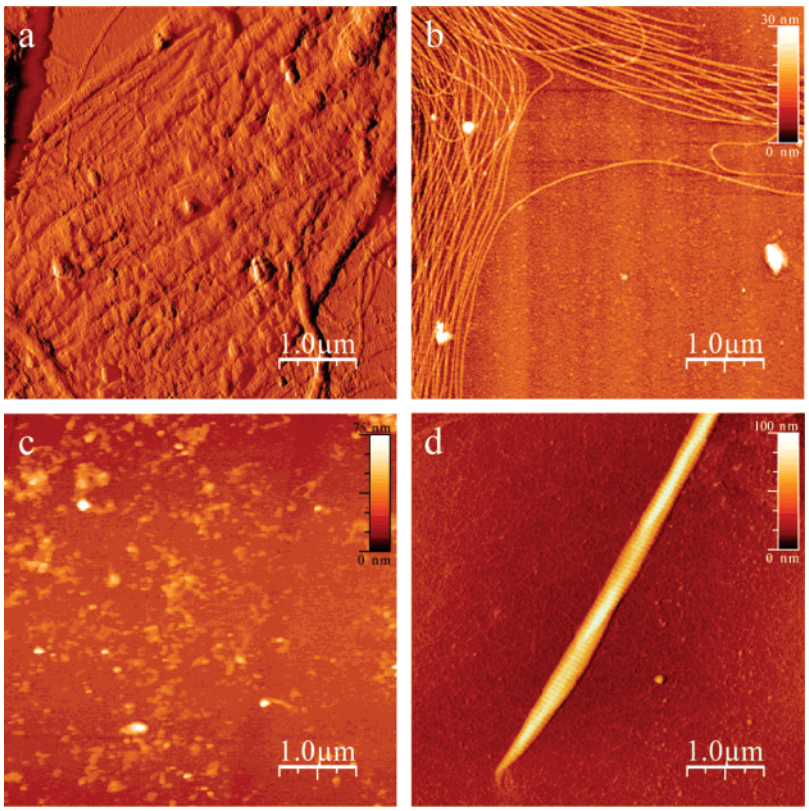

Figure 2. AFM images of *Chondrosia reniformis* sponge collagen after 7 days of suspending in 0.1 M acetic acid (a, deflection image), 0.1 M Tris/HCl buffer solution pH 7.4 (b, height image) and 0.1 M NaOH (c, height image). Panel d, (height image) shows a reassembled fibril of bovine type I collagen.

Table 1. Amino Acid Composition of Bovine Collagen Type I and Collagen of the Marine Glass Sponge *Hyalonema sieboldi*, and Two Previous Measurements on *Chondrosia reniformis* Sponge Collagen

amino acid	bovine type I collagen [25]	*Hyalonema* collagen[12]	*Chondrosia* collagen[7]	*Chondrosia* collagen[20] [a]
Ala	11.1	6.2	6.2	7.7
Arg	5.0	4.8	4.6	4.9
Asx	4.3	10.7	10.4	9.5
Cys	0.0	0.2	0.0	0.3
Glx	7.1	9.3	9.0	10.3
Gly	33.5	24.5	30.6	18.9
His	0.6	1.6	0.4	1.1
H-LPro	10.4	6.9	9.8	2.4
Ile	0.9	3.8	2.5	5.0
Leu	2.3	4.3	3.6	6.2
L-HLys	1.0	1.5	1.6	4.3
Lys	2.3	2.0	0.7	2.8
Met	0.6	0.4	0.2	1.4
Phe	1.2	2.2	1.8	3.2
Pro	12.0	6.5	6.3	5.4
Ser	3.3	4.7	4.5	4.2
Thr	1.7	5.6	4.3	4.7
Tyr	0.2	1.2	0.6	2.1
Val	2.6	4.1	2.9	5.5

[a] The last column summarizes the composition of the collagen used in this study.

dermis of sea cucumber *Cucumaria frondosa* 1:2000,[34] spine ligament of sea urchin 1:5000).[26,35] To compare, tropocollagen shows an aspect ratio of 1:200 and reassembled fibrils of bovine type I collagen about 1:50.[36] Maybe the remarkable aspect ratio of *Chondrosia* collagen is an important structural feature essential for the reported stiffening effects of the sponge's mesohyl.[37]

Alkali treatment of skin is a common method for collagen extraction from connective tissue. Alkali-treated type I collagen lost the ability to form fibrils at neutral pH;[38] however, the triple helical conformation and the helicity of the collagen molecule were maintained throughout the period of the alkaline treatment. When *Chondrosia* collagen was treated in 0.1 M NaOH, hydrolysis also occurred and clear solutions were obtained after a few days. In this case, AFM imaging showed unequal fragments ranging from nanoparticles to short fibrils or huge aggregates (Figure 2c). Also reassembling in physiological salt solutions at neutral pH could not be observed. Figure 2d shows an AFM image of a bovine type I fibril reassembled from tropocollagen by the method described and serving as a reference.

Regarding the insolubility in acid media, similar properties are known for aged type I collagen and for the recently discovered collagen of the marine glass sponge *Hyalonema sieboldi*. In Table 1, the results of two previous measurements on the amino acid composition of *Chondrosia* collagen are compared to that of bovine type I collagen and the collagen of *Hyalonema sieboldi*.[8]

The differences in the results comparing the *Chondrosia* collagens, primarily concerning the reduced values for glycine and hydroxyproline, can be explained by either major non-triple-helical sections in the analyzed collagen molecules or by impurities of glycoproteins, which are known to be strongly associated with collagen.[20] The comparison of the three sponge collagen compositions reveals remarkable similarities in nearly all amino acid contents. Furthermore, noticeable deviations from bovine type I collagen are mainly limited to certain amino acids, whereby the general composition is maintained.

Table 2. FT-IRAS Spectra Peak Position and Assignments for *Chondrosia reniformis* Sponge Collagen and Fibrillar Calf Skin Type I Collagen[39]

peak wave number [cm^{-1}]	assignment
3330	amide A ($\nu_{as}NH_2$)
3070	amide B (ν_sNH_2, νCH)
3000-2800	νCH_x
1660, 1634	amide I (νCO)
1555, 1537	amide II (δNH, νCN)
1453, 1403	δCH_2, γCH_2
1341, 1281, 1239, 1205	amide III (νCN, δNH, νCC)
1081, 1051, 1032	νCO

On the basis of the results of the solubilization experiments in this study, single fibrils were favored for further purification and analysis. Therefore, suspending in 0.1 M Tris/HCl buffer pH 7.4 solution for 7 days at 4 °C was chosen for standardized preprocessing. Filtering these suspensions removed insoluble constituents before lyophilization, dialysis, and repeated lyophilization were performed. The chemical properties of this lyophilizate were analyzed by Fourier transform infrared reflection−absorption spectroscopy (FT-IRAS). FT-IRAS is a well-known method which has been used to study changes in the secondary structure of collagen and collagen denaturation, cross-linking, thermal self-assembly, and comparison with gelatin.[39] We also used this highly sensitive method to investigate the isolated collagen of *Chondrosia reniformis*. Spectra for this collagen and a calf skin collagen standard (Fluka) were compared to each other in order to elucidate differences in protein secondary structure (Figure 3). The results obtained by FT-IRAS examination show that collagen derived from sponge exhibited spectra very similar to those from calf skin collagen.

The wide scan infrared spectra show the typical bands such as N−H stretching for the amide A, C−H stretching for the amide B, C═O stretching for the amide I, N−H deformation for the amide II and N−H deformation for the amide III band.[40] The wave numbers at which major peaks occurred for both collagens are summarized in Table 2.

The clear correlation of nearly all peak positions and band intensities is impressive in view of the entirely different phylogenesis and origin of both collagens. Furthermore, this result demonstrates that the chemical properties of the *Chondrosia* collagen are not negatively affected during the purification procedure. The comparison of the chemical data in Table 1 and Figure 3 does not permit a clear distinction to be made between the sponge collagen and bovine type I collagen which could explain the insolubility of these fibrils.

The organization of the moderately separated *Chondrosia* collagen fibrils was observed by parallel TEM and AFM imaging. Positive staining was recognized to be best suited to visualize the arrangement of fibrils and fibers, and negative staining was more suitable at higher magnifications of single fibrils. The positively stained fibrils show a characteristic banding pattern of alternating dark and light segments (Figure 4 a). The single fibrils are joined into thick bundles by a close side-by-side assembly, in which the banding pattern is completely retained. Using high-resolution AFM imaging, the single fibrils making up the bundles can be identified (Figure 4b). Furthermore the deflection image shows the twisted but regular assembly and banding pattern which is characteristic for the fibrils. The diameter of the thick fiber in Figure 4b taken from the corresponding height image has been calculated to be in the range 100−150 nm. Higher magnification in atomic force microscopy reveals an impressive topography of the fibrils as

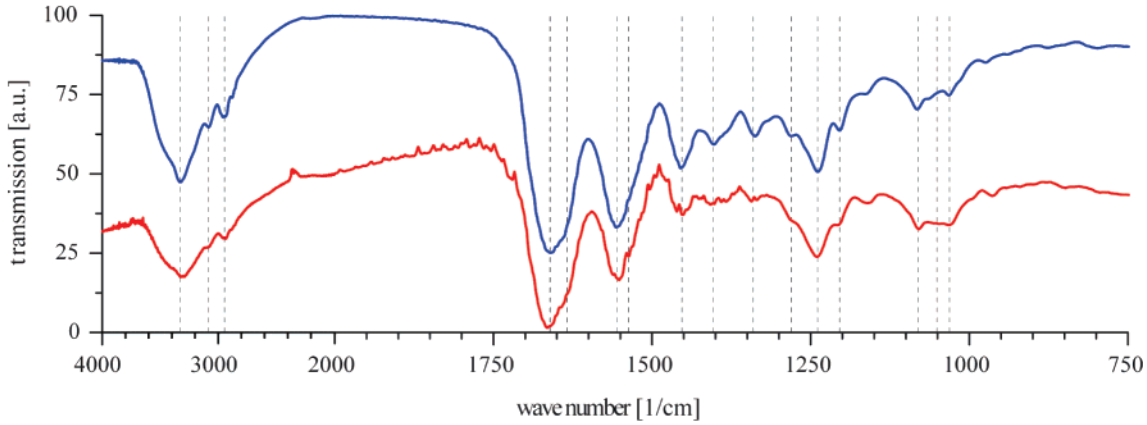

Figure 3. Infrared spectra of purified *Chondrosia reniformis* sponge collagen suspended in 0.1 M Tris/HCl buffer solution pH 7.4 (lower, light line). The comparison to the spectrum of fibrillar calf skin type I collagen (upper, dark line) shows a clear correlation in the position, intensity, and shape of the peaks.

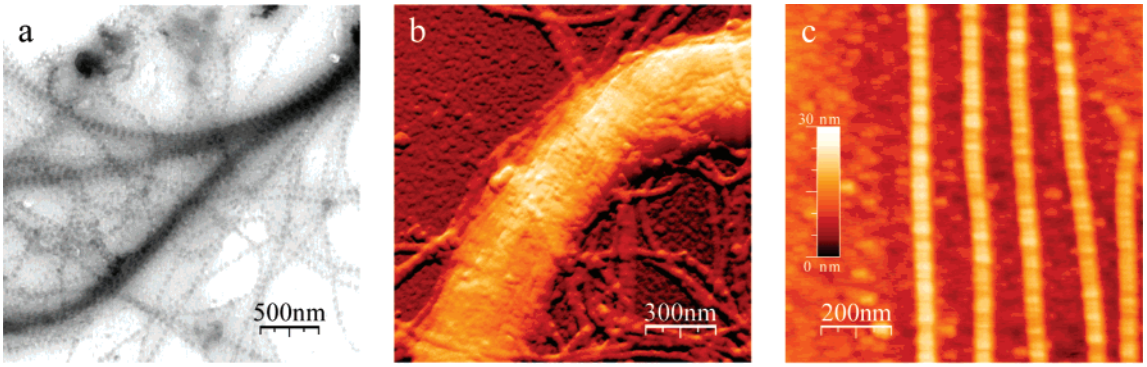

Figure 4. TEM image (a) of positively stained *Chondrosia* collagen fibrils shows the directed lateral association of the single fibrils as well as high-resolution AFM imaging (deflection image, b). The height image in panel c shows the unique banding pattern of five slightly separated *Chondrosia* collagen fibrils.

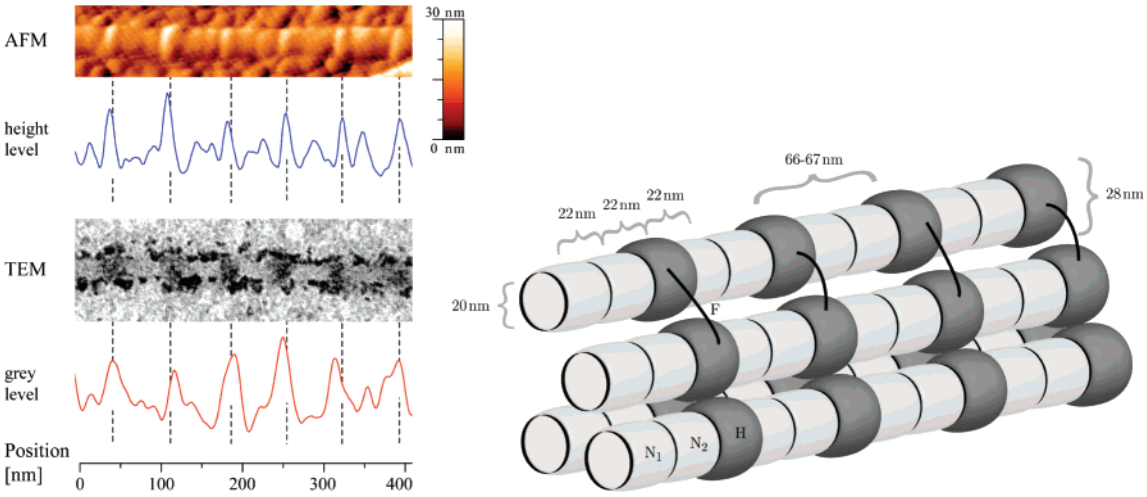

Figure 5. The comparison of a representative AFM section and related longitudinal height level as well as TEM image and related gray level shows the similarity of the banding patterns. The schematic diagram on the right side illustrates the three-dimensional organization of the *Chondrosia* collagen fibrils supplemented with morphological data from AFM investigations and data on staining behavior obtained by means of TEM. The major repeating dark bands (H) observed under TEM are thick relative to the two following segments (N_1, N_2) of light interband regions. The formation of collagen bundles is facilitated by directed side by side arrangement of the single fibrils assisted by fine filaments (F).[27]

shown in Figure 4c. To the best of our knowledge, this parallel arrangement of five single fibrils allows the first insight on the fibrils of *Chondrosia reniformis* sponge collagen using AFM and showing the multiperiodic banding pattern.

Detailed information on the morphology and especially the banding pattern of the separated *Chondrosia reniformis* sponge collagen fibrils was collected by measuring transversal and longitudinal height profiles of AFM images. In Figure 5 a representative section and the according profile are given. Two groups of peaks can be identified corresponding to the course of the observed height level. Along the fibril one characteristically thick segment (protrusion) of about 28 nm in diameter is followed by two equal thinner and closer conjoined segments (interband) of about 20 nm in diameter, respectively. The average distance between the protrusions is about 67−69 nm. Between two following peaks of the interband regions or

between a protrusion and adjoined interband region about 21−23 nm was measured. The average step height between the protrusions and the interband regions was calculated to be about 4 nm.

For comparison, a representative section of a negatively stained sponge collagen fibril with adapted magnification is shown in the same figure. The course of the gray level in the longitudinal axis reveals the alternation of relatively short, dark segments followed by a broad, light segment. A clear partition of the light segment into two equal parts, as is feasible using AFM, is not possible in this case. Regarding the peak positions of the height level and gray level, the correlation of the dark segments to the protrusions identified by AFM is unambiguous. A similar phenomenon, where dark-stained bands correspond to protrusions on the collagen fibril, was observed by Lin and Goh.[13] Our results strengthen the claim of these authors that dark bands observed by TEM after negative staining do not necessarily represent gap zones. However, our data does not refute the Hodge−Petruska model, which is widely accepted for most types of collagen.[41] Considering the combination of the new topographical data obtained by AFM and the results of TEM investigations, now we can provide an advanced model as illustrated on the right in Figure 5, based on the former scheme of the organization and morphology of the *Chondrosia reniformis* sponge collagen provided by Garrone et al.[27]

Conclusion

A crucial part when studying the morphology of collagen fibrils is the separation of single fibrils. In this study we pointed out the characteristic insolubility of *Chondrosia* collagen but developed a procedure for purification of the isolated sponge collagen and proper conditioning for further analysis. Similarities in the amino acid composition of bovine type I collagen and *Chondrosia* collagen recurred in nearly identical infrared spectra. Owing to the developed purification procedure, for the first time high-resolution AFM imaging has been used to describe the ultrastructure of *Chondrosia reniformis* sponge collagen. The method revealed the morphology of thick collagen fibers assembled from single fibrils each showing a characteristic banding pattern. By performing a parallel AFM−TEM approach, it has been shown that the periodic protrusions of the fibrils elucidated by AFM correspond directly to the periodic dark bands along the length of the fibril observed in TEM images.

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