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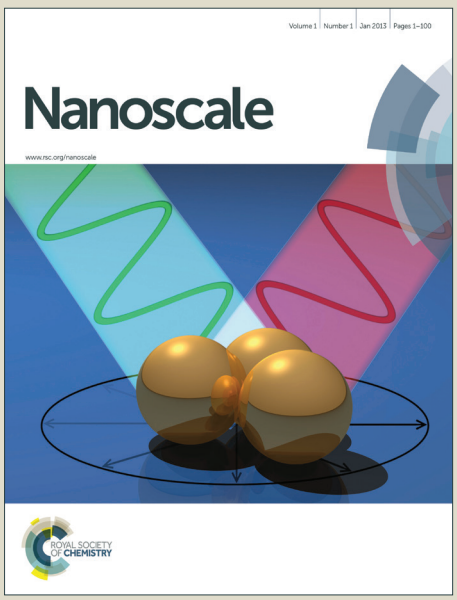

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The Ultrastructure of Type I Collagen at Nanoscale: Large or Small D-Spacing Distribution?

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Abstract

D-spacing is the most significant topographic feature of type I collagen fibril, and it is important to our understanding of the structure and function in collagens. Traditionally, the D-spacing of type I collagen fibril was considered as a singular value of 67 nm, but recent works indicated that the D-spacing values had a large distribution up to 10 nm measured with atomic force microscopy. We found that this large distribution of D-spacing values was mainly resulted from image drift during measurement. The D-spacing was homogeneous in a single type I collagen fibril. Our statistic analysis indicated that the D-spacing values of type I collagen fibrils exhibited only a narrow distribution of 2.5 nm around the value of 67 nm. In addition, D-spacing values of the collagen fibrils were nearly identical not only within a single fibril bundle, but also in different fibril bundles. Measuring the D-spacing value of collagen may provide important structural information in many research areas, such as collagen related diseases, construction of molecular model of collagen, and collagen fibrogenesis.

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1. Introduction

Collagen is the most abundant protein in animals, and it exists in skin, bone, and most other tissues throughout the body, maintaining the integrity of these tissues[1,2]. The basic building block of collagen is tropocollagen, which is 300 nm long and is comprised of three helical polypeptide chains, each chain containing Gly-X-Y triplets (X is usually proline and Y is usually hydroxyproline)[3-5]. Tropocollagens are further assembled into high ordered collagen fibrils[6,7]. The gaps formed between tropocollagen ends and the overlap zones produce a repeated surface structural pattern called D-periodicity[7, 8].

The D-periodic spacing (or D-spacing) is the most significant topographic feature of collagen fibril. Measuring the D-spacing is important to our understanding of the ultrastructure of collagen fibril. Much attention has been devoted to elucidate molecular architecture of collagen. The microscopic topography is a requisite reference when constructing the detailed molecular model of collagen fibril[6,9,10]. The D-spacing is also related with the mechanism of collagen fibrillogenesis[7]. Moreover, the ultrastructure of collagen is closely related with its functions[11]. For example, it was considered that the alterations of D-spacing in collagen fibrils were related with some diseases[12-15]. Thus, due to the relationship of structure and function in collagen, researchers have been prompted to study the ultrastructure of collagen with various methods, such as transmission electron microscopy[16-19] and atomic force microscopy (AFM)[20-24].

Traditionally, a singular D-spacing value of 67 nm has been widely accepted.

Since AFM was introduced in researching collagen, the ability of 3-dimensional imaging made AFM to be very powerful in studying surface morphologies of this macromolecule. Recently, some measurements with AFM have revealed a distribution of D-spacing values up to 10 nm in collagen from various tissues, including dermis, tendon, and bone[7, 25, 26]. Such a distribution is rather large, for in these reports, the smallest D-spacing value in some collagen fibrils could only be less than 4/5 of the largest value. Taking this point into account, the detailed molecular model of how tropocollagens connect with each other to construct collagen fibrils may be reconsidered.

In our previous work, we investigated the degradation process of collagen by collagenases with AFM[27, 28]. The morphological properties of collagens were studied and D-spacing values were measured. We found that when calculating the D-spacing values in AFM images, there were indeed large variations in accordance with previous reports. However, with further research, we noticed that the variations of these measured values were mostly resulted from image drift. Even different D-spacing values could be measured in the same collagen fibril when the scanning angles were different.

Drift usually leads to slight distortion to the images, resulting in moving, elongation, or compression to the images. This effect could be from either thermal (both internal and external) or instrumental reasons (such as scanner creep). As the piezoelectric ceramic of the AFM scanner is sensitive to the temperature, slight temperature changes would introduce thermal drifts. The distortion of the image

caused by image drift depends on scanning angle and scanning velocity.

Here we report our work about the measurement of the D-spacing values in type I collagen. We found that the D-spacing values measured with AFM have only a small distribution, instead of a large distribution in previous reports.

2. Results and discussion

AFM is a powerful tool in researching the ultrastructure of collagen fibril due to its ability of 3-dimensional imaging with high resolution. In our work, we did notice that the D-spacings from different fibrils were not a singular value when measured in AFM images. For example, the D-spacings of the three collagen fibrils in Fig. 1 were 65 nm (fibril 1), 64 nm (fibril 2), and 62 nm (fibril 3), respectively. Statistical analysis of D-spacing values on different collagen fibrils exhibited a distribution of 10 nm. This result seems reasonable, for other literatures also reported that there is a 10-nm distribution in the D-spacing values of collagen fibrils[7, 25, 26].

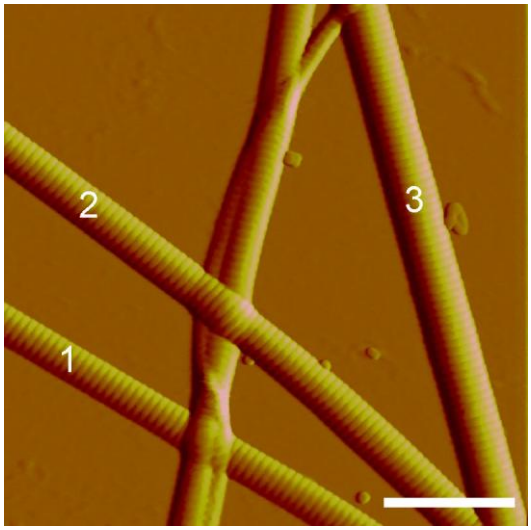

Fig. 1 AFM image of collagen fibrils with different D-spacing values. Different collagen fibrils were indicated with numbers. Scale bar: 1 μm.

In later research, the scanning angle was adjusted to get a better view of the samples, and we surprisingly noticed that the measured D-spacing value of the same collagen fibril often changed significantly. For example, in Fig. 2, when the scanning angles were different, the measured D-spacing values from this collagen fibril were 67 nm (Fig. 2A), 68 nm (Fig. 2B), 69 nm (Fig. 2C), and 71 nm (Fig. 2D), respectively. We carried out experiments on different collagen fibrils, and found that this phenomenon was ubiquitous.

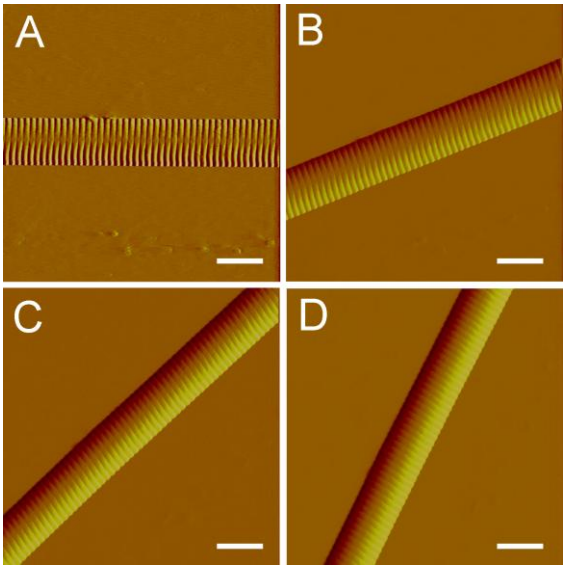

Fig. 2 AFM images of collagen fibrils with different angles with respect to horizontal scanning axis. Scale bar: 0.5 μm.

Some potential factors would introduce technique errors that resulted in such a discrepancy in measured D-spacing values of the same collagen fibril. The extension of scanner in one direction is not a function of a straight line with applied voltage, and this is called the nonlinearity effect. Assuming the AFM had been correctly calibrated,

the effect of nonlinearity could be much reduced. Thus the drift in images, which is one of the most common and unavoidable factors for introducing the technique error in measurements[29-32], might be the most probable reason. And this could be recognized from the elongation or compression of the images. Elongation or compression is a kind of scanner artifact that usually resulted from image drift. Topographic feature recorded on the low scanning axis are more sensitive to the image drift than that on the fast scanning axis. The elongation or compression of images caused by drift effect are commonly seen in AFM researches, and usually result in only subtle changes in the images, thus they are often ignored by the researchers. But when precisely measuring the dimensions of small samples in an image, the influence of these effects would be much clear.

Considering the working principle of AFM, the samples were scanned by the AFM tip line by line along x-axis (fast scan axis), and the line data assembled into an image. The time needed to acquire an image could be calculated by scanning rate and scanning line. For example, in the case of scanning an image with 512 lines at a rate of 1 Hz with Multimode AFM, it needs about 8.5 min to get the full image. Within such a long time, drift in image would introduce small technique errors when measuring the precise length of a sample, especially on the direction of slow scan axis. Various methods are often used to minimize the effect of drift to the images, including stabilizing the environmental conditions or increasing the scanning rate. However, sometimes this effect could be minimized but can not be completely avoided. The piezo is sensitive to the environmental conditions, and even slight

temperature changes would introduce drift to the images, resulting in the compression or elongation of the frame.

Because of the existence of image drift, it is understandable that there is variation in the measured D-spacing values of the same collagen fibrils. Considering the scanning velocity of the tip along the slow scanning axis (y axis) is much slower than that along the fast scanning axis (x axis). The effect of AFM drift on the slow scanning axis is relatively obvious[33], while the effect on the fast scanning axis is much smaller, usually negligible[34]. Thus when measuring the D-spacing value, the scanning angle should be adjusted to let the tip scan along the collagen fibril (i.e., collagen fibril along the fast scanning axis), to minimize the technique error and get data that are much closer to the accurate values.

The effect of fibril orientations on the measured D-spacing values might depend on the elongation or compression to the image. In an elongated image, the collagen with larger angle with respect to the fast scanning direction tends to have larger D-spacing value. In a compressed image, the collagen fibril with larger angle tends to have smaller D-spacing value.

Type I collagen from mice tail tendon and porcine neck dermis were also checked for comparison to test the possible effect of image drift on the D-spacing measurement (Fig. S1, S2). The effect of image drift could be recognized from the images of both samples. When the scanning angles were adjusted and the effect of image drift was reduced, we found that the type I collagen fibrils from both sources shared similar D-spacing values with bovine collagen fibrils. As the amino acid

residue sequences of type I collagen subunits from different sources shared very high similarity, it is reasonable that the structures of these type I collagens are similar.

Then we checked whether the D-spacing was homogeneous with in a single collagen fibril. We imaged various collagen fibrils with different lengths ranging from about 10 μm to 180 μm. One example was shown in Fig. 3, in which the length of this collagen fibril indicated by white arrows (Fig. 3A) was estimated to be about 100 μm. We imaged different parts of this collagen fibril (Fig. 3B, C, D), and the D-spacing values were estimated. The D-spacing from different locations of this collagen fibril showed a singular value of 67 nm. Similar results were obtained from other observed collagen fibrils. Thus we concluded from our results that the D-spacing value within a single collagen fibril was homogeneous.

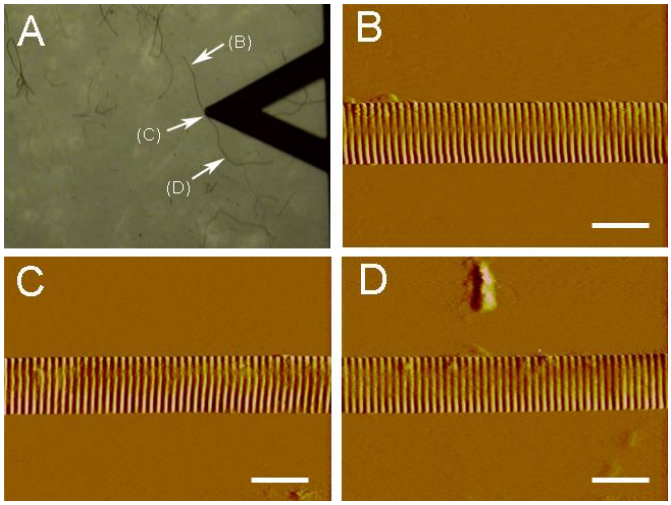

Fig. 3 (A) A whole single collagen fibril pointed by the tip cantilever viewed under light microscopy. (B, C, D) AFM images of the single collagen fibril at different locations indicated with white arrows in (A). Scale bar: 0.5 μm.

At least 123 single collagen fibrils were imaged and their D-spacing values were

measured. After statistical analysis, we found that the D-spacing value of our results ranges from 66 nm to 68.5 nm (Fig. 4A). The distribution of the D-spacing value was as small as only 2.5 nm, which was inconsistent with a large distribution up to 10 nm in previous reports. The collagen fibrils with the D-spacing values ranging between 66.5 nm and 67.5 nm (1 nm distribution around 67 nm) account for 85% of the total fibrils. In addition, the relationship of D-spacing values and fibril width was also analyzed (Fig. 4B). There seemed no apparent correlation between the D-spacing values and fibril widths.

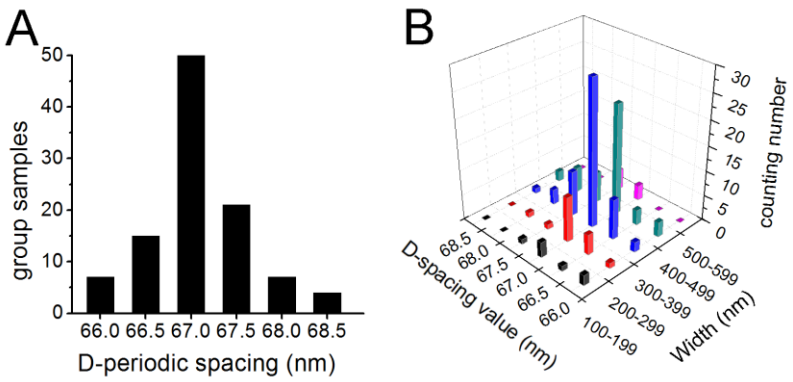

Fig. 4 (A) Histogram of D-spacing values measured from collagen fibrils (1 nm bin size). (B) 3D histogram that shows the distribution of D-spacing values in relation with the width of collagen fibrils. Each bar indicates the counting numbers of collagen fibrils, and fibrils with different width (100 nm bin size) are indicated by different colors.

A singular D-spacing value of 67 nm has been widely accepted, and the statistical analysis of the D-spacing values of the type I collagen is rare in traditional work. Only recently, reports suggested that the distribution of the D-spacing values was as large as 10 nm after statistic analysis[7, 25, 26]. If such a large distribution was true,

it would alter our knowledge of collagen. For example, some detailed molecular models of fibrillar structures of type I collagen were built upon the singular D-spacing value of 67 nm[9, 10, 35, 36]. However, in the papers which reported large distribution of D-spacing values, the technique errors brought by image drift was not considered.

In our work, after carefully quantitative analysis, we found that there was indeed a distribution of measured D-spacing values of collagen fibrils. However, surprisingly, our statistical analysis showed a distribution of only 2.5 nm, much smaller than that in previous reports. As for this small D-spacing value distribution, some potential factors may contribute to this distribution, such as the hydration of the collagen sample and the slight drift along the fast scanning axis. Future investigations are needed to clarify these effects. However, in general, the measured D-spacing value of type I collagen exhibited only a small distribution of 2.5 nm rather than a large one of 10 nm.

In addition to the image drift, some other potential factors might also contribute to the distribution of measured D-spacing values. There is inevitably a limit in the resolution of an image. Assuming an image with dimension of 3.5 × 3.5 μm and 512 points in each direction, each pixel represents about 6.8 nm. When there are fewer pixels defining the image, it will result in larger errors. In this case errors would occur either in direct profile section analysis or in two-dimensional Fast Fourier Transform (2D FFT) image processing in the corresponding image. This effect will be more obvious when the collagen fibrils are not parallel to the x-y plane.

Groups of parallel collagen fibrils would associate into bundles in the body, and randomly oriented collagen fibril bundles are then organized together in tissues.

Several models about the nucleation and growth of collagen fibrils have been developed[37-41]. Whether there is heterogeneity in D-spacing values of collagen fibrils within/between collagen fibril bundles is important to our knowledge of how these collagen fibril bundles are formed. Recent work reported that the D-spacing values within a collagen fibril bundle are similar, and fibrils from different bundles exhibited different D-spacing values[7]. This viewpoint that D-spacing values differ at bundle level creates direct constraints to the existing models of how collagen fibril bundles are formed[7]. However, it could be noticed from those works that the results were based on the measurements which overlooked the effect of the technique errors brought by different scanning angles.

The drift would bring about slight compression or elongation to the images, and if the collagen fibrils are aligned in parallel, the effect of image drift to these collagen fibrils is usually similar. As indicated in Fig. 5A, the collagen fibrils indicated by white arrows shared same angles (28 °) with respect to the horizontal scan direction. The D-spacings of these collagen fibrils measured from this image shared a same value of 68 nm. As we know, the collagen fibrils in a bundle are largely parallel arranged, thus it is understandable that the D-spacings of the collagen fibrils within a bundle share similar values.

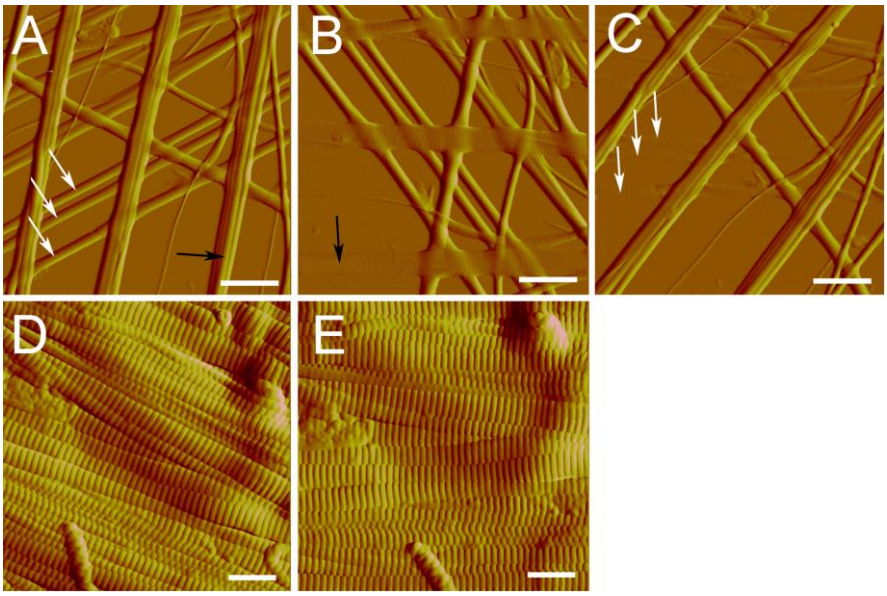

Fig. 5 (A) AFM images of collagen fibrils with different orientations. Black and white arrows indicated two groups of parallel aligned collagen fibrils. (B) and (C) are the same location with (A), only adjusting the scanning angles to let the fast scanning axis along the orientation of the fibrils indicated by black and white arrows respectively. (D) AFM image of a collagen fibril bundle. (E) is the same bundle with (D), with the adjustment of scanning angle to let the fast scanning axis largely along the orientation of the fibril bundle.

Based on our findings, different scanning angles would result in the variations in the measured D-spacing values. The most probable reason might be the measured lengths of collagen fibrils with different angles with respect to the horizontal axis have different sensitivity to the image compression or elongation. The black arrow in Fig. 5A indicated another group of collagen fibrils which had different angle to the fibrils indicated by white arrows. The D-spacing values of these collagen fibrils were measured to be 64.5 nm, different from the D-spacing values (68 nm) measured from

the collagen fibrils indicated by white arrows. Different collagen fibril bundles usually have different angles with respect to horizontal axis in an image, and this might be the reason why the D-spacing values in different collagen fibril bundles are different.

When the scanning angles were adjusted to let the tip scanning along the collagen fibrils, the technique errors could be minimized and more accurate results could be measured. We found that after the scanning angles were adjusted, the D-spacing values indicated by white and black arrows shared the same value of 67 nm (Fig. 5B, C). Apart from the parallel single fibrils, similar results could be observed in the case of collagen fibril bundle. For example, the D-spacing values of the fibrils with the bundle in Fig. 5 changed from 67.5-68.5 nm (Fig. 5D, before scanning angle adjustment) to 66.5-67.5 nm (Fig. 5E, after scanning angle adjustment). There was small variation in the measured values in this bundle, maybe because these collagen fibrils in the bundle were not completely parallel aligned. Experiments on different collagen fibril bundles showed similar results.

Our results indicated that the D-spacing values in different collagen fibril bundles are similar, which is in contrary to the previous works. The viewpoint in published papers that collagen fibrils from different bundles have different D-spacing values largely results from technique reasons. The constraints brought by this viewpoint to the model of collagen fibril bundle formation should be cleared.

In general, by adjusting the scanning angles of AFM, the influence of image drift was much reduced and more accurate D-spacing values of type I collagen were

measured in this work. Recent studies with AFM analyzed large amounts of collagen fibrils, and suggested a distribution of D-spacing values up to 10 nm. In our experiments, we carefully checked the D-spacing values of type I collagen with AFM, and found that this 10-nm distribution was largely resulted from image drifts. The D-spacing values exhibited only a small distribution of 2.5 nm around the value of 67 nm, and the D-spacing values were similar in different collagen fibril bundles. Measuring the D-spacing value of collagen is important to the understanding of the collagen ultrastructure, and provides important structural information in many research areas, such as tissue engineering, collagen related diseases, construction of molecular model of collagen, and collagen fibrogenesis. AFM is a powerful tool in characterizing the nanoscale morphological features of macromolecules except for collagens. Precisely measuring the dimensions and topographic features of macromolecules with AFM needs several requisites: stabilizing the environmental conditions, correctly calibrating the instrument, and minimizing the technique errors. Technique error brought by image drift is very common in AFM experiments, and it was sometimes overlooked by researchers. Our work of measuring D-spacing values of type I collagen may provide some implications to researchers when studying other macromolecule samples in the future.

3. Experimental

Type I collagen (from bovine Achilles tendon) was purchased from Worthington Biochemical. Collagen samples were suspended in distilled water and then collected

and deposited onto freshly cleaved mica. Samples were rinsed with distilled water to remove debris and then air-dried. Before imaging, calibration of the AFM was performed according to the Manual provide by the manufacturer. Imaging was carried out in contact mode or ScanAsyst mode in air (512 × 512 pixels) using a Multimode Nanoscope VIII AFM (Bruker AXS, Germany) equipped with a cantilever of NSC11/AIBS (with a spring constant of 5.5-22.5 N m^{-1}, from MikroMasch, used in ScanAsyst mode) or NP-S10 (with a spring constant of 0.32 N m^{-1}, from Bruker AXS, used in contact mode). The deflection set point in contact mode was optimized to minimize the contact force. The width and D-spacing values were measured with the Nanoscope software. In the statistical analysis work, only single collagen fibrils were selected.

4. Conclusion

We measured the D-spacing values of type I collagen fibrils with AFM and found that the D-spacing is homogeneous within a single collagen fibril. Statistic analysis indicated that the D-spacing exhibits only a narrow distribution of 2.5 nm around the value of 67 nm, inconsistent with the recent works which considered the D-spacing values have a large distribution up to 10 nm. The large distribution of D-spacing values in previous works mainly resulted from image drifts. Moreover, D-spacing values of collagen fibrils are nearly identical, both in a single fibril bundle and in different fibril bundles. Measuring the D-spacing value of collagen may provide important structural information in many research areas, such as, collagen related

diseases, construction of molecular model of collagen, and collagen fibrogenesis, and this work may also provide some implications to researchers when studying other macromolecule samples in the future.

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