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Nanostiffness of Collagen Fibrils Extracted from Osteoarthritic Cartilage Characterized with AFM Nanoindentation

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Osteoarthritis (OA) is a prevalent and deliberating joint disorder, which acts as the leading cause for the disability and poor quality of life of the middle-age and elderly people. It is generally believed that OA is degeneration of articular cartilage. However, the debate remains on the role of subchondral bone in pathogenesis of OA. In this study, the nanostiffness of collagen fibrils from articular cartilage in patients with entirely different bone metabolism, that is, OA, osteoporosis (OP), and health, was quantitatively measured with AFM nanoindentation technique. It was found that the stiffness of individual collagen fibril from healthy cartilage was 2.67 ± 0.12GPa under ambient condition and 11.24 ± 0.74MPa in hydrated state respectively. The collagen fibrils were softer in osteoporosis (OP) group (ambient: 1.64 ± 0.12GPa; hydrated: 8.59 ± 0.59MPa). By contrast, the extracted fibrils from OA cartilages were stiffer (ambient: 4.65 ± 0.25GPa; hydrated: 17.26 ± 1.77MPa). The results obtained demonstrated that the collagen fibrils extracted from OA patients are stiffer than those from healthy patients, and therefore the nanomechanical characterization of extracted collagen fibrils may be a promising way for early diagnosis of OA.

Keywords: Articular cartilage, Atomic force microscopy, Collagen fibril, Nanoindentation, Stiffness

Introduction

Collagen fibrilis a basic structural component of articular cartilages (AC), and their biomechanical properties are one of the determinants for the mechanical performance of AC. The individual collagen fibrils disruption, which occurs at the onset of naturally occurring osteoarthritis (OA), could be a major attribute to the breakdown of AC collagen meshwork (1, 2). OA does not have significant symptoms at its early stage, and once the collagen meshwork breaks down, the only clinical way to fully cure OA is total knee arthroplasty, which is expensive and painful. A reliable method for early diagnosis of OA will therefore be greatly helpful in its clinical treatment.

A number of studies have investigated the nanomechanical properties of collagen fibrils, while most of them focused on type I rather than type II collagen fibrils from AC (3–17). Recently, the nanomechanical properties of AC collagen fibrils were examined in various animal models and human OA specimens via atomic force microscopy (AFM) nanoindentation (18–20). Stiffening of collagen fibrils meshwork was identified as an early biomarker for the onset of OA. Thus far, minimal attention has been paid to the nanomechanical properties of individual collagen fibrils in pathogenesis and pathophysiology of OA.

Attempts have been made to address the *in-situ* nanomechanical behavior of collagen fibrils of AC tissues in human OA specimens (21). The nanomechanical tests were performed on the individual collagen fibrils inside sectioned pieces of cartilage tissue under dehydrated state using AFM tips with a radius of 10 nm. Stiffening of collagen fibrils was observed in cartilages suffering from OA. Unfortunately, AC collagen fibrils inside tissues are randomly packed and the orientation of collagen fibrils might interfere with the *in-situ* measurement. Moreover, it can be difficult to measure the mechanical properties of individual collagen fibrils inside cartilage tissues *in vivo,* because any small vibrations during the AFM nanoindentation may cause significant errors during the measurement. Nanomechanical characterization of individual collagen fibril extracted from tissues and using the measured mechanical properties as a biomarker for early diagnosis of OA may be a

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promising way, if a suitable extracting protocol is established and the differences in mechanical properties of OA and healthy fibrils remain significant after the extraction process. In our previous work (21), an abnormally rich Ca^{+} element was found in the bone-cartilage interface, which indicates that the stiffening of OA fibrils may be due to the calcification of fibrils. In this study, collagen fibrils extracted from healthy, OA, and OP cartilages were investigated using AFM nanoindentation under both ambient and hydrated conditions with the purpose of validating the diagnosis of OA based on the mechanical properties of extracted fibrils. SEM and micro-CT analyses were performed to study the morphology and structure of both extracted fibrils and subchondral plates to further understand the pathology of OA.

Experimental Details

Human Specimens Collection and Preparation

All experimental procedures were approved by the Institutional Review Board of the authors' institute (Ref Nr: UW 09-368). The intact AC specimens were obtained from femoral heads of two healthy donors (35–45 years old, Nanfang Medical School, Guangzhou, China) and three osteoporotic hip fracture patients (70–96 years old, Queen Mary Hospital, Hong Kong). The osteoarthritic AC specimens were collected from patients (34–70 years old), undergoing total hip or knee replacement surgery at the authors' institute.

All AC specimens were stored in saline-soaked gauze, and thawed at 4°C for 24 h and were then cut into small pieces with area around 2 mm^2. The collagen fibrils were extracted from hyaline AC tissues using the protocol as fellow: (1) the small pieces of AC samples were immersed in the extraction buffer with the initial sample-to-buffer volume ratio at 1:5, and were then dispersed with a homogenizer (T10 Basic UltraRTurrax@, KA, Germany). During this process, the extraction buffer was gradually added in until the volume ratio of sample:buffer reached 1:15 (2). The dispersed sample in the buffer was placed into a Himac CR-GIII centrifuge (Hitachi Koki Company, Japan), and centrifuged for 30 min at 4°C. After centrifugation at 27000 g, the supernatant was collected (3). The aforementioned procedures were repeated twice. Then the supernatant was centrifuged at 115000 g and the final pellets were collected in a centrifuge tube and stored in double distilled H_2O (DD H_2O) with 2% sodium lauryl sulfate (SDS) (Wako, Japan) at 4°C for subsequent evaluations. The extraction buffer was composed of 7.5 mM of sodium chloride (Fisher Scientific, UK), 0.1 mM of ethylenediaminetetraacetic acid disodium salt dihydrate (ETDA) (Sigma-Aldrich, US), 3 mM of 6-aminohexanoic acid (Sigma-Aldrich, US), 5 mM of benzamidine (Sigma-Aldrich, US), 0.1 mM of phenylmethanesulfonly fluoride (PMSF) (Sigma-Aldrich, US), and 5 mM of N-ethylmaleimide (NEM) (Sigma-Aldrich, US). A Schematic diagram of the extracting process is shown in Fig. 1.

SEM

Evaluation of collagen fibril nanostructure was performed under a Hitachi S-4800 scanning electron microscope (SEM). Collagen fibril pellets were thawed at room temperature, and then dropped on the glass slides coated with polylysine (Sigma-Aldrich, US). The droplet, together with the glass slide, was dehydrated in a desiccator with humidity at approximately 20% and the dried sample was then gently washed by deionized distill water to remove the SDS precipitant. Afterward, the sample was dried in the desiccator again. The images were captured approximately 20 mm apart from each other to avoid duplicated measurements. A total of 350 collagen fibrils were in each sample, and the diameters of all the fibrils were measured.

Micro-CT

Subchondral plate refers to a small piece of condensed bone that mainly consists of hydroxyapatite between the cartilage and cancellous bone, and it responds to the transmitting loads from the cartilage into the underlying cancellous bone. The subchondral plate directly contacts the cartilage, and thus it should influence the biological and biochemical conditions, for instance, ion concentration, inside cartilage. The Micro-CT experiments were performed on the OP and OA subchondral plates, respectively. A micro-CT scanner supplied by Belgium (SkyScan 1076, Kontich, Belgium) was employed and the scanning parameters were as follows: 18 μm isotropic voxel size, 55 kV voltage, 109 μA current, 200 ms integration time, and 4000 projections.

AFM Imaging and Nanoindentation

Nanowizard II Bio-AFM (JPK Company, Germany) was adopted in this study. The nanomechanical properties of collagen fibrils were examined at ambient and hydrated states, respectively. AFM imaging was initially performed to visualize the position of the sample under the semi-contact mode for the ambient condition and the contact mode for the hydrated state; then, the AFM tip was moved to the desired position for indentation on the fibrils. The AFM tips of both NCHR-20 (spring constant 42 N/m) and CONTR-20 (spring constant 8 N/m) (NanoWorld Innovative Technology, US) were employed. For the tests performed under the hydrated state, the dried samples were re-hydrated in deionized distilled water for 45 min, and then the glass slide was placed in a tissue culture dish filled with deionized distilled water for the AFM imaging and nanoindentation. More than 60 AFM nanoindentation tests were performed on randomly selected positions on various collagen fibrils under the ambient and hydrated states, respectively. Because the accuracy of the mechanical properties measurement is very dependent on the quality of the experimental data, only the data sets with insignificant experimental noise caused by external vibration during the nanoindentation were selected for the final data analysis. Therefore, a total of 50 sets of experimental data under ambient conditions and a total of 20 sets of data in the hydrated state were selected for the final analysis, respectively. The radii of all tips were at around 8 nm under SEM evaluations.

The nanoindentation tests were performed by directing the AFM tip toward the sample with a designed schedule; the piezoelectric driven movement of the clamped based of the tip δ, which served as the input, and the vertical photodiode signal D, which served as the output, were recorded during the test. The spring constant k of the used tip was further calibrated with the thermal noise method, and the cantilever sensitivity A was calibrated by performing indentation tests on a clean glass surface and linearly fitting the obtained $\delta - D$ curves beforehand. The deflection of the cantilever at its free end is $\delta = AD$ and therefore the force

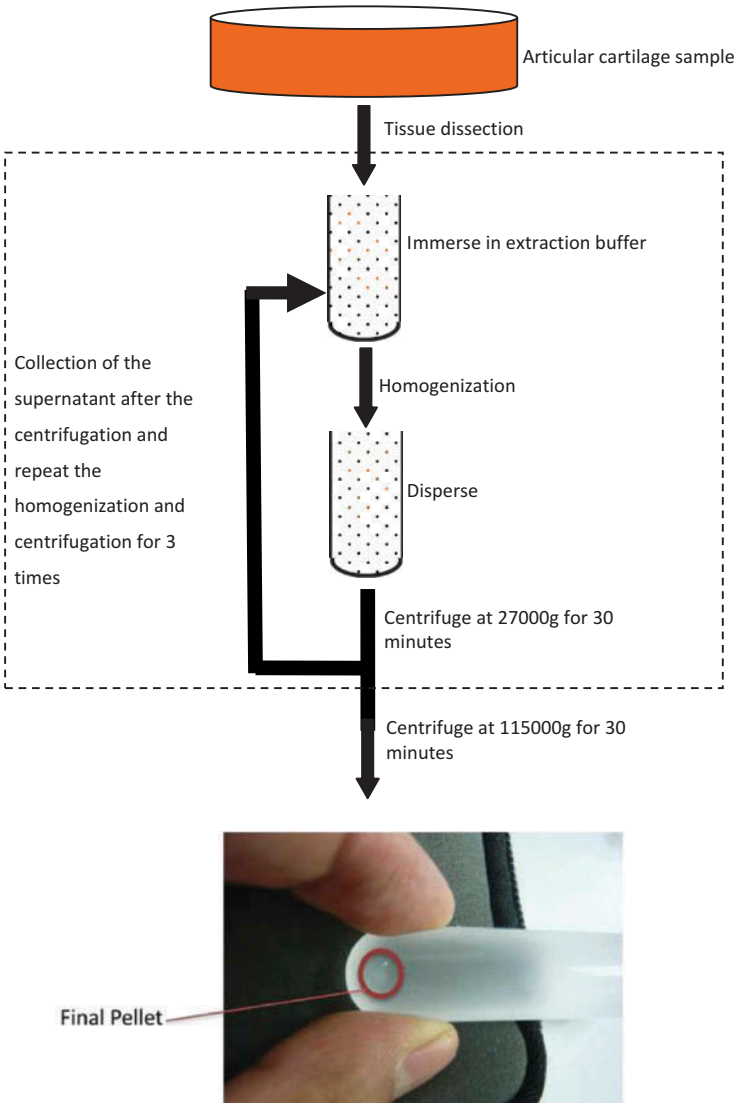

Fig. 1. Schematic diagram shows the extraction procedure of collagen fibrils from hyaline articular cartilages for AFM nanoinentation test.

applied onto the samples is $F = kAD$, and the indentation depth of the tip into the sample is $d = \delta - AD$. The obtained $\delta - D$ curve therefore can be converted into the typical force versus the displacement curve (F-d curves) of the sample. It is assumed that the AFM tip used in this study has a pyramidal shape, and a modified Hertz model for a four-sided pyramidal indenter was used for data analysis by fitting the converted F-d curves with the following equation (22):

$$F = 0.7453 \frac{E}{1 - v^2} d^2 \tan a, \tag{1}$$

where the v is the Poisson's ratio of the sample and a is the apex angle of the tip. A typical 3D-image of a collagen fibril and a typical F-d curve after conversion are shown in Fig. 2. As the diameter of the collagen fibrils is typically over 60 nm, only the first few nanometers after the initial solid contact in the recorded data were adopted for curve fitting to avoid possible substrate effects. Based on our observation, Eq. (1) fit the experimental data from the initial solid contact region very well. It should be noted here that the collagen fibrils are viscoelastic, and, therefore, the fitted E value may not be the elastic modulus that represents the purely elastic deformation. However, a materials parameter represents the resistance of viscoelastic and elastic deformation of collagen fibrils at nanoscale; therefore, we defined the E measured here as "nanostiffness." The Poisson's ratio of collagen fibrils was determined to be 0.5 thereafter.

Statistics Analysis

The data were presented as mean ± standard error, and also 95% confidence intervals. The relationship between the nanostiffness of individual collagen fibrils of intact AC and their diameters were analyzed using Pearson's correlations. SPSS 20.0 (Chicago, IL, USA) was used with the statistical significance set at 0.05.

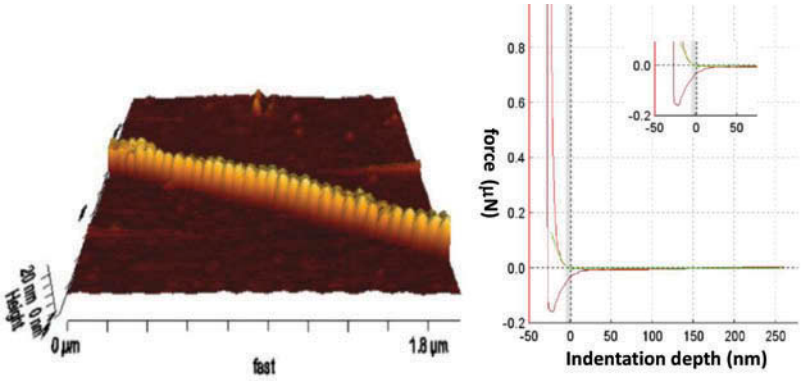

Fig. 2. A typical 3D image of one collagen fibril (left) and a typical *F-d* curve of AFM nanoindentation performed (right). The green line on the right figure is the fitting curve of the experimental data with equation (1), and the fitting is good at the beginning of solid contact.

Results

Under SEM examination, the distributions of the extracted collagen fibrils' diameters of intact AC specimens were similar among four healthy donors and three osteoporotic hip fracture patients. However, only thinner collagen fibrils were extracted from OA cartilages as compared with healthy and OP groups (Fig. 3). The height profile along the longitudinal direction of one collagen fibril was also plotted under AFM images to examine its structural characteristics. It was found that the peak-to-peak distance, that is, the D-periodicity of the collagen fibril, was in the range of 60–70 nm (Fig. 4).

The nanostiffness of collagen fibrils from healthy AC specimens was depicted under AFM nanoindentation. The 95% confidence intervals for the nanostiffness of collagen fibrils from the samples H1-1, H1-2, H2-1, and H2-2 were 2.19–3.23, 1.03–1.76, 2.17–3.17, and 2.07–3.02 GPa under ambient condition, and 9.40–15.10, 8.56–13.93, 8.02–14.85, and 6.16–10.72 MPa under hydrated condition, respectively. No statistically significant correlations were detected between the nanostiffness of the collagen fibrils and their diameters under either ambient ($r^2 = 0.07$, $p = 0.143$) or hydrated condition ($r^2 = 0.05$, $p = 0.820$).

As compared with healthy specimens, the collagen fibrils from OA cartilage yielded a higher nanostiffness under both ambient (4.65 ± 0.25GPa) and hydrated (17.26 ± 1.77MPa) conditions (Fig. 5). Yet, the collagen fibrils were softer in the OP group (ambient: 1.64 ± 0.12GPa; hydrated: 8.59 ± 0.59MPa).

The microstructure of subchondral plate harvested from OA and OP patients were also examined with micro-CT, and the results are shown in Fig. 6. It was found that the subchondral plate harvested from the OA patient is much porous than those from OP patient.

Discussion

This study presented an approach of AFM nanoindentation on the extracted collagen fibrils to detect the altered nanomechanical properties of hyaline AC tissues in different disease conditions, for example, osteoporotic hip fracture and OA. First, the D-periodicity of the extracted collagen fibrils in the range of 60–70 nm agreed well with previous studies (21, 23). The elastic moduli of collagen fibrils (1.03–3.23 GPa) were also compatible with the data measured under *in-situ* AFM nanoindentation (1.99–3.91 GPa) (21). Our findings further confirmed the previous observations of collagen fibrils stiffening in OA cartilage (18, 21).

According to the SEM results, we found that the extract collagen fibrils' diameter was in the range of 40–60 nm, and this is relatively smaller than that of the collagen fibrils in normal AC we measured previously, which is in the range of 80–100 nm (21). The difference could be a result of the nature of this extraction protocol. The centrifugation was employed to release the fibrils from the tissues and the collagen fibrils with smaller diameters might be easily isolated. No significant association was detected between the fibrils' diameters and their nanostiffness. In this sense, the nanomechanical properties of the thin collagen fibrils after extraction might be the representative of the all AC collagen fibrils.

The nanostiffness of the extracted collagen fibrils measured here is in the same order of magnitude of previous reported data for the extracted type I collagen fibrils (Table 1). For example, our data are compatible with Heim et al.'s study, which showed that the stiffness of collagen fibrils from tissues of the inner dermis of sea cucumber was in the range of 1–2 GPa (4). In this study, the nanostiffness measured under hydrated state was in the range of 8.02–15.10 Mpa. To our knowledge, these are the first batch of data reported for AC collagen type II collagen fibrils under a hydrated state. By contrast, the stiffness of collagen type I fibrils from bovine Achilles tendon is in the range of 1.1–1.3 Mpa under hydrated condition, and that of collagen fibrils from human dentin is in the range of 30–60 Mpa. Our data are also in the same order of magnitude of collagen type I fibrils. Interestingly, both others' and our study showed that the stiffness of collagen fibrils measured under the hydrated state is nearly 1000 times smaller than that measured under ambient condition (Table 1). It is believed that the rehydration process might significantly soften the collagen fibrils after artificially regaining the water content.

The nanostiffnesses of collagen fibril meshworks in human OA cartilage specimens were once reported in the range of 15 to 142 kPa (18), that is, $\sim 10^3$ times smaller than that the data of individual collagen fibrils in this study. Such discrepancy could not be easily explained with the differences in test protocols and

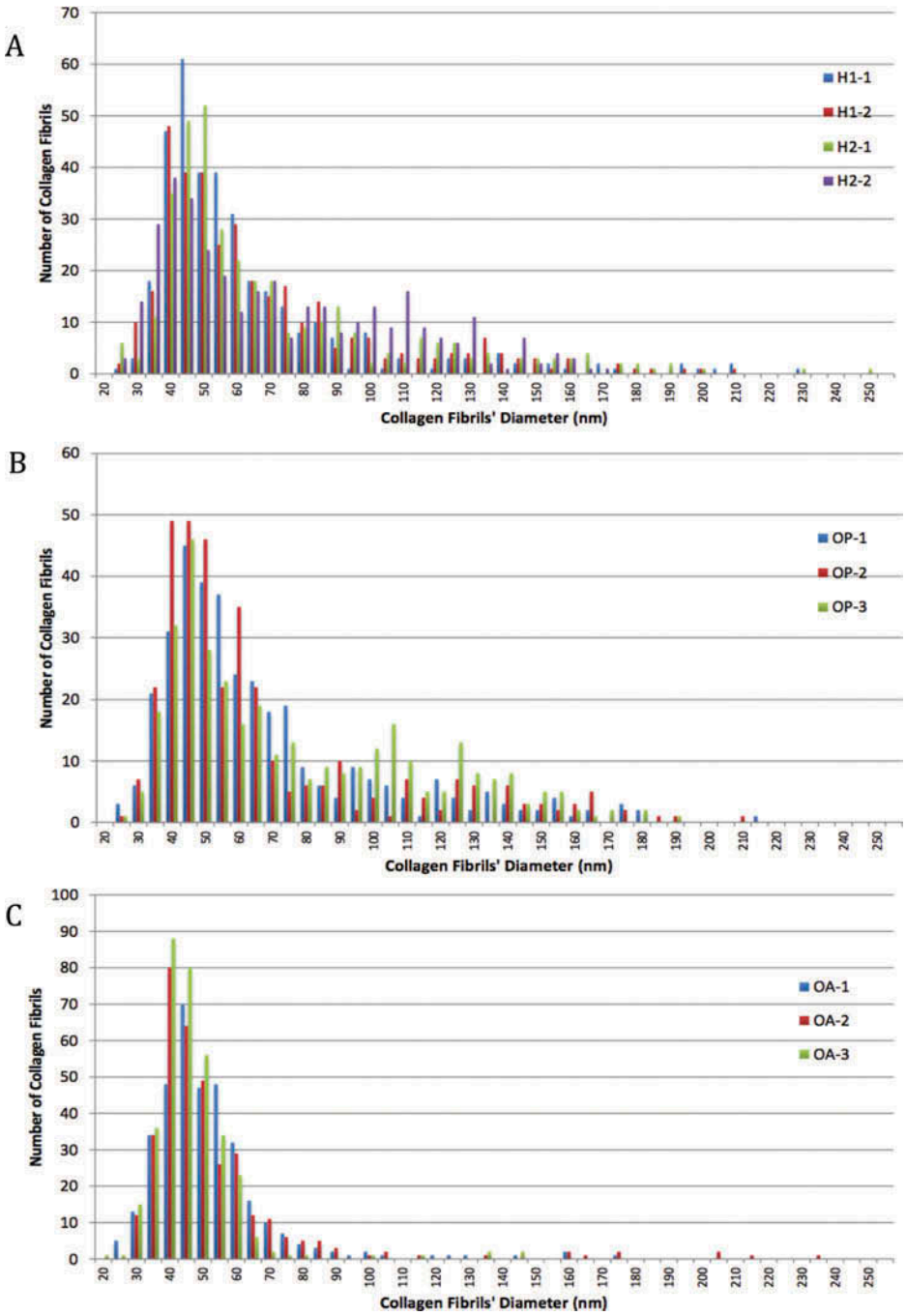

Fig. 3. Distribution of the extracted collagen fibrils from articular cartilages of healthy donors (Panel A, H1, and H2), osteoporotic hip fracture (Panel B, OP-1, 2,3) and osteoarthritis patients (Panel C, OA-1, 2, 3). As compared with healthy (A) and OP groups (B), only thinner collagen fibrils were extracted from OA cartilage (C).

conditions. Actually, a previous study (21) and attempted to measure *in-situ* properties of the collagen fibrils in AC under hydrated condition, yet the mechanical properties they measured could be the bending modulus of collagen fibrils, as well as the complex modulus of the entire collagen fibrils meshwork and, therefore, cannot be compared with the stiffness of individual collagen fibril measured in present study.

It is well known that OA is the result of the mechanical failure of the collagen meshwork in articular cartilage. Therefore, monitoring the nanostiffness of AC collagen fibrils could be a promising way for early detection of OA (18). Recently, the stiffening of collagen fibrils from OA cartilage of human beings has been demonstrated (21). Yet, it is not easy to probe the nanomechanical properties of AC *in vivo* because any small vibrations during the AFM nanoindentation test may cause severe reduction of the accuracy of measurement. In our previous work, small pieces of tissues were harvested from AC, and nanomechanical tests were performed on individual fibrils inside

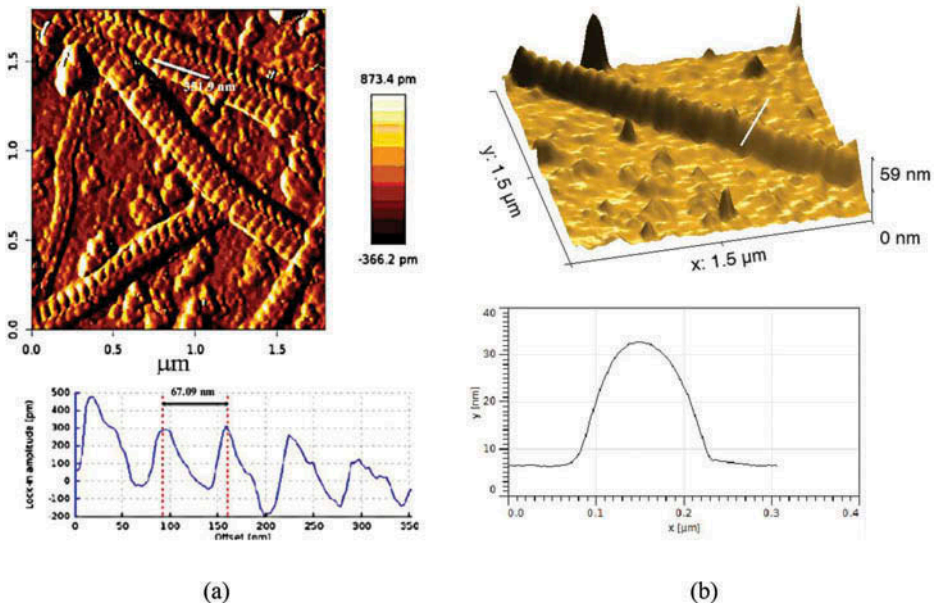

Fig. 4. (a) A typical AFM image showing the extracted collagen fibrils and the height profile of one collagen fibril along the longitudinal direction. The D-periodicity of the collagen fibril is about 67 nm; (b) 3D-image of a collagen fibril, the height of collagen fibrils was measured to be around 30 nm.

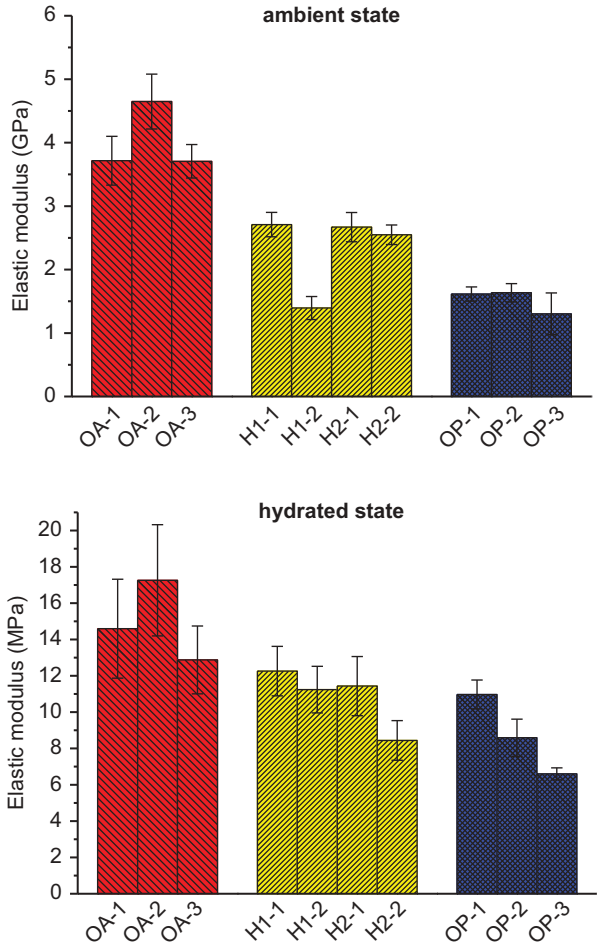

Fig. 5. AFM nanoindentation tests of the extracted collagen fibrils were performed under both ambient and hydrated conditions.

Fig. 6. Micro-CT image of OP and OA subchondral plate. It can be found that the OA subchondral plate is much porous than the healthy plate.

Table 1. Summary of nanomechanical properties of individual collagen fibrils in literature.

References	Type of Collagen (Source)	Biomechanical Test	Hydration Condition	Elastic Modulus	Remarks
Eppell et al., 2006 (24)	Type 1 Collagen (Sea cucumber)	Tensile	Ambient	0.4GPa to 0.6GPa	The large range of elastic modulus was due to large range of strain rate, $\varepsilon \sim 0.05$ to 0.3
Heim et al., 2004 (4)	Type 1 Collagen (Sea cucumber)	Nano-Indentation	<45%	1GPa to 2GPa	
van der Rijt et al., 2006 (7)	Type 1 Collagen (Bovine Achilles Tendon)	Tensile	Ambient	2GPa to 7GPa	
van der Rijt et al., 2006 (7)	Type 1 Collagen (Bovine Tendon)	Tensile	Immersed in PBS	0.2GPa to 0.5GPa	
Wenger et al., 2007 (8)	Type 1 Collagen (Rat Tail Tendon)	Nano-Indentation	various hydration statuses	3.75GPa to 11.5GPa	The large range of elastic modulus may be related to various hydration statuses.
Balooch, et al., 2008 (25)	Type 1 Collagen (Human Dentin)	Nano-Indentation	Immersed in DD H_2O	30Mpa to 35Mpa	Indentations were performed at the gaps of collagen fibrils D-periodicity
I Balooch, et al., 2008 (25)	Type 1 Collagen (Human Dentin)	Nano-Indentation	Immersed in DD H_2O	60Mpa	Indentations were performed at the overlaps of collagen fibrils D-periodicity
Shen et al., 2008 (5)	Type 1 Collagen (Sea Cucumber)	Tensile	31% to 60%	0.36GPa to 1.6GPa	The large range of elastic modulus may be related to large range of humidity. Tensile tests were performed under relatively high strain rate, $\varepsilon \sim 4$
Yang et al., 2008 (9)	Type 1 Collagen (Bovine Achilles Tendon)	3-Point Bending	Ambient	1.0GPa to 3.9GPa	
Yang et al., 2008 (9)	Type 1 Collagen (Bovine Achilles Tendon)	3-Point Bending	Immersed in PBS	0.07GPa to 0.17GPa	
Yadavalli, et al., 2010 (26)	Type 1 Collagen (Self-Assembled)	Nano-Indenation	Ambient	1.9 ± 0.5GPa	
Yadavalli, et al., 2010 (26)	Type 1 Collagen (Self-Assembled)	Nano-Indenation	Hydrated	1.2 ± 0.1Mpa	

the sectioned tissues *in-situ* (21). Unfortunately, because collagen fibrils are randomly oriented and packed in the AC extracellular matrix, the accuracy of the measurement was limited by the substrate effects of indentation and the uncontrolled fibril orientation. Therefore, the method employed in this study, which is to extract small amount of collagen fibrils from patient and measure their nanostiffness, should be much more nondestructive, feasible, and reliable. In the present study, it was demonstrated that the mechanical difference between individual collagen fibrils from OA and healthy patients will be kept even after extraction and,

therefore, provides a promising method of OA early diagnosis: the articular biopsy could be obtained from patients suffering from knee pain and submitted for early diagnosis and/or precise prognostication of OA using the present method.

The collagen fibrils are viscoelastic, and the viscoelastic effects might influence the accuracy of our measurements as the Hertzian contact model employed for data analysis is based on the assumption that tip-sample contact is purely elastic (27–30). In this study, although a relative fast loading rate was employed and the only the recorded curve at the very beginning of elastic contact region was utilized for the Hertzian fitting to minimize the viscoelastic effects during the measurement, the nanostiffness measured here may not represent the purely deformation of fibrils. Moreover, the dehydration and/or rehydration process of collagen fibrils adopted in this work may introduce internal stress in the specimens because the sample volume was changed and, therefore, might also influence the accuracy of the measurement. These limitations should be taken into consideration when interpreting the data presented here, and further improvement in the measurement technique is desired.

The Micro-CT results in Fig. 6 clearly show that the subchondral plate of OA cartilage is much porous than those of OP, and this is believed to be the possible reason for the stiffening of collagen fibrils in OA. The Ca^{+} ions in cartilage should mainly come from the subchondral plate. In OP cartilage, it should have a diffusion gradient of Ca^{+} ions decrease from the bone-cartilage interface to the superficial region of cartilage, and therefore the level of calcification of collagen fibrils inside the cartilage should also follows this trend. It was determined that the nanostiffness of collagen fibrils decreased from the bone-cartilage interface to the superficial region in healthy AC (21). However, for the OA cartilage, the porous structure of subchondral plate largely increased the surface area of the plate and thus increased the Ca^{+} ions release rate from bone to cartilage. This may cause the abnormal richness of Ca^{+} ion in cartilage and should lead to a higher level of fibril calcification in OA AC than those in OP cartilage. The collagen fibrils extracted from OA cartilage, therefore, are stiffer that those from healthy and OP cartilage. The key assumption of this hypothesis is that the concentration of Ca^{+} ions will change the mechanical properties of collagen fibrils, and therefore it is suggested that more studies should be performed to investigate the effects of calcification on collagen fibrils' mechanics.

Conclusion

In this study, the nanomechanical properties and the microstructure of fibrils extracted from healthy, OA, and OP AC were systematically investigated. It was found that the nanostiffness of the extracted collagen fibrils from OA AC was relatively higher than those of fibrils from healthy AC, and this implies the feasibility of the presented AC collagen fibril extraction protocol and the potential diagnostic values of *in-vitro* AFM nanoindentation for OA. The Micro-CT analysis together with the AFM nanoindentation results revealed a possible pathology of OA. The microstructure of subchondral plate became porous, and therefore the largely increased specific surface area of subchondral plate, which enables the Ca^{+} ion to be released much easier from the plate to cartilage in OA AC than in normal AC, results in a higher level of fibril calcification in OA AC. The calcified fibrils are brittle and hard and might have a higher chance of being broken than healthy fibrils under imposed stress, which leads to the initiation of OA.

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