J Mater Sci: Mater Med (2008) 19:249–256
DOI 10.1007/s10856-006-0049-2

Supramolecular structure of human aortic valve and pericardial xenograft material: atomic force microscopy study

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Received: 10 February 2006 / Accepted: 20 November 2006 / Published online: 28 June 2007

Abstract Pericardial tissue (bovine or porcine), chemically stabilized with glutaraldehyde (GA), is widely used in cardiovascular surgery in the form of bioprosthetic valves. GA reacts with tissue proteins and creates inter- and intramolecular cross-links, resulting in improved durability. However, tissue calcification and mechanical damage are still unresolved problems. The purpose of this study was to examine the surface topography of normal human aortic valve and GA-stabilized porcine pericardium tissue in order to gain comparative insight into supramolecular structure of both tissues. The analysis was focused on morphologic evaluation of collagen constituents of the tissues. Atomic force microscopy working in the contact mode in air was employed in the study. Considerable diversity in the spatial orientation of collagen fibrils for the human aortic valve and pericardial tissue were observed. It was found that different forms of collagen fibril packing, i.e. dense and "in phase" or loose, could have an impact on the collagen D-banding pattern. Stabilization with GA introduced significant changes in the surface topography of collagen fibrils and in their spatial organization on the tissue surface. Strong disturbance in the fibril's D-spacing was observed. It was also suggested, that the observed structural changes at the supramolecular level might make an important contribution to the progressive damage and calcification of the tissue. The presented results demonstrate that the AFM method can be useful for non-destructive structural characterization of heart valves and bioprosthetic heart valve material.

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Introduction

Heart valve bioprosthetics give a great advantage for improved treatment of valve diseases. Significant progress in the field was made in the early 1990s, the subject however remains in its infancy and many problems still have to be resolved [1–3]. Primarily, our understanding of the normal heart valve structure and function is limited. A thorough knowledge of supramolecular structure of heart valves seems to be a prerequisite to construct the desired valve bioprostheses.

Currently available valve replacement materials include, among others, pericardial glutaraldehyde-fixed tissues. Fixation with glutaraldehyde (GA) preserves the tissue against rapid enzymatic and chemical degradation in the body and improves its mechanical properties. However, the use of GA as the tissue-stabilizing agent is associated with several problems including progressive structural deterioration, calcification and early mechanical failure. The origin of these problems is multifactoral and it is still under investigations [4, 5].

According to the literature data [6–10], most of the structural investigations of human heart valves and heart valve bioprostheses were performed by scanning electron microscopy and light microscopy. Atomic force microscopy (AFM) represents a novel and increasingly important method for non-invasive examination of the surfaces of different biological materials under slightly altered

physiological conditions. AFM enables morphological studies revealing nanometer-scale details of biological objects. Unlike electron microscopes, samples do not need to be stained, coated, dehydrated or frozen. Measurements can be performed in ambient liquid or gas environments [11].

Usually, bioprosthetic heart valves are fabricated from GA-fixed bovine pericardium or porcine aortic valve, however porcine pericardium is also investigated as a potential bioprosthetic material [12–16]. In our study, we used porcine pericardium for two main reasons. First, in comparison to bovine pericardium, the porcine pericardium is of more regular thickness, collagen content is very close to the bovine one (71.8 ± 2.1% and 75.0 ± 1.2%, respectively), the water content is slightly higher (89.5 ± 0.8% and 88.4 ± 0.6%, respectively) and porcine pericardial tissue is richer in cross-linked type I and III collagens [16]. Second, we used porcine pericardium with the serosal layer removed from the sample. Such material is structurally more homogeneous allowing for restriction of the analysis to the fibrosal layer as the main component of the sample. Moreover, using thin and more homogeneous tissue-sample one can expect a potential reduction in the amount of glutaraldehyde required to achieve complete fixation, thus possibly limiting the adverse effects associated with the use of GA.

The aim of the present study is to examine surface topography of normal human aortic valve and glutaraldehyde-fixed porcine pericardium tissue, using AFM imaging system. Collagen is the principal structural component of the bioprosthetic heart valves. The analysis has been focused on morphologic evaluation of collagen constituent.

Materials and methods

Preparation of human aortic valve

Human aortic valve was collected post mortem from 28 years old woman, who died from non-cardiac disease, according to the protocol of multiple organ procurement from cadaver donors [17]. Briefly, during first 12 h after the death, the skin of the cadaver was disinfected using betadine alcoholic solution. Then the sterile disposable blades were used for the skin and sternocostal cartilages dissection. After opening the pericardial cavity the heart was excised using sterile scissors. Then the heart was immersed in sterile physiologic saline and transported to sterile room for preparation of valve leaflets. The preparative procedure was conducted under the saline, also in sterile conditions. The excised leaflets were placed in PBS saline buffer, pH 7.4 and kept at 4 °C. The central part of each valve was excised and subjected to AFM measurements.

Preparation of porcine pericardium

Pericardium tissues from hearts of 5–6 month old pigs were obtained fresh from slaughter and subsequently transported to the laboratory in buffered physiological saline solution (pH = 6.5) at 4 °C. According to the pericardial tissue selection for bioprosthetic heart valves described by Simionescu et al. [18], the area that overlies the left ventricle of the heart was choosen for the investigations. Fatty tissues and sections with heavy vasculature or attached ligaments together with the serosal layer were gently removed from each sample. The 5 mm × 10 mm rectangles were excised from each sample.

Chemical treatment of pericardium tissue

Chemical stabilization procedure of the pericardium tissue was performed using 0.2% solution of glutaraldehyde in 0.2 M phosphate buffer (pH = 6.5). The reaction was held at 4 °C for 2 h.

The tissue samples were dissolved in an excess of reacting solution, 2 mL/mg of dry tissue.

Conditions of the stabilization procedure were in agreement with the procedures described previously in literature. According to the earlier investigations by Jayakrishnan et al. [19], it was found that the use of 0.2% GA in 0.2 M phosphate buffer (pH 6.5) for 2 h leads to the maximum number of cross-links and to the highest thermal stability of collagen membranes. Higher concentrations of GA or longer time of cross-linking does not cause an increase in these parameters.

The tissues assigned to the chemical modifications were taken from the same pericardium tissue. After chemical treatment the tissues were rinsed three times with distilled water and then kept in phosphate buffer at 4 °C until the measurements could be made.

Water content

The wet weight of the tissue was measured before they were vacuum dried at 313 K for 0.5 h, and then the dry weight was obtained. The water content of the tissue was calculated as (wet weight—dry weight)/dry weight, and expressed in g/g. The water content of the native, GA-treated pericardium tissues as well as human aortic valve are equal to 4.09 ± 0.05 g/g, 4.28 ± 0.05 g/g and 4.21 ± 0.05 g/g, respectively.

AFM measurement

AFM imaging was performed using the NanoScope E, Digital Instruments, USA, working in the contact mode. The microscope was equipped with OTR8 probe (Veeco

NanoProbe™). The spring constant of the used V-shaped cantilever was 0.15 N/m while its length was 200 μm. The applied constant forces were about 10 nN. We used AS-12 "E" scanner therefore the maximum size of images in both: X and Y horizontal directions was 13 μm. The maximum height (Z-limit) was 3.8 μm. A single measurement (one frame: 512 × 512 pixels) lasted about 300 s. Two standard AFM signals were registered: the signal corresponding to the topography of the sample (Height) and the differential signal (Deflection), which is useful for direct observation. The samples were rather soft therefore the images were obtained with lateral and height resolution of about 10 nm and 1 nm, respectively. The software package WSxM, Nanotec Electronica, Spain, was used for image processing.

The valves were divided into small rectangles (of about 5 mm × 10 mm). Then the fragments were rinsed with distilled water and placed onto clean glass microscope slides. The measurements were performed in air. To obtain stable images, the tissue samples had to be gently dried on air, at room temperature, until the excess of water had evaporated from the samples' surfaces. The drying process lasted about 30 min. Longer drying did not cause considerable changes in the surface topography. According to the earlier findings presented by Jastrzebska et al. [20], after short drying on air at room temperature, collagen fibrils imaged on tissue surface were still in a hydrated state. Therefore, the properties of collagen fibrils in the investigated tissues are expected to be close to the normal, native state. However, some effects caused by drying will be described in Section "Results and discussion". Both sides of aortic valve, i.e., inflow and outflow surfaces, were imaged by AFM.

Results and discussion

Human aortic valve

Figure 1 shows the AFM Height (a, c) and Deflection (b, d) images of the central part of the fibrosa layer of the human aortic valve. The aortic valve is composed of three layers: ventricularis, spongiosa and fibrosa [21]. The ventricularis layer is located on the inflow surface of the valve (the side of the valve which faces the direction of the blood flow). It contains abundant amount of elastic fibers. Elastic fibers allow the tissue to withstand repeated deformation and reformation during the heart valve opening and closing cycle. The spongiosa layer consists of loosely organized connective tissue with highly hydrated proteoglycans as the dominant matrix components. The fibrosa layer is composed predominantly of densely packed collagen fibrils. Collagen fibrils are responsible for the most of the mechanical strength of the valve. As shown in Fig. 1b, collagen fibrils form thick irregular bundles, which are aligned in a disordered, tangled manner.

The image taken at higher magnification shows areas with the densely packed collagen fibrils aligned in ordered manner (Fig. 1d). The collagen fibrils are characterized by a transverse D-banding pattern consisting of subsequent grooves and ridges. Figure 1e presents an axial profile taken along the marked line on the fibril surface in the Height image (Fig. 1c). The mean distance between the neighbouring grooves is equal to (56 ± 6) nm, i.e. exhibits lower value in comparison to the most frequently cited D-period for the type-I collagen fibrils as equal 67 nm [22–25]. This result can correspond to the dense packing of fibrils observed in aortic valve. As it is shown in Fig. 1d, the characteristic feature of such dense packing is that collagen fibrils are aligned with the same groove/ridge phase. The observed alignment "in phase" can imply some forms of intermolecular coupling. However, an explanation of these interactions requires further studies.

In contrast to the fibrous layer of the leaflet, the ventricularis layer shows relatively smooth surface with poorly distinguished fibrilar structure. Figure 2 shows Height (a, c) and Deflection (b, d) images of the aortic valve ventricularis layer. The image taken at higher magnification (Fig. 2d) reveals some fibril-like objects. They are supposed to be collagen fibrils with many protein/lipoprotein adhesions that overlap the characteristic D-banding pattern. Ventricularis is rich in elastin fibrils. They are much thinner than collagen fibrils. Elastin fibrils together with proteoglycans and other endothelial layer components form an amorphous ground substance in which collagenous fibrilar structures are deposited. In our measurements, the structure of the highly hydrated ventricularis has been partially disturbed owing to short time drying of the sample surface before experiment. Endothelium is the most vulnerable component of the valve leaflets.

Native and GA-stabilized porcine pericardium tissue

Figure 3 shows the Deflection (a, b) and the Height (c) images of the native porcine pericardium tissue at two different magnifications. As the serosal layer has been removed from the tissue surface, the fibrosa layer is well presented. Collagen fibrils are running in different directions and they are loosely arranged on the tissue surface. The thick collagen fibril bundles are observed only sporadically. At higher magnification (Fig. 3b), collagen fibrils show characteristic D-banding pattern. Figure 3d presents an axial profile describing the surface topography of the collagen fibril taken along the marked line shown in the Height image (Fig. 3c). The mean distance between the

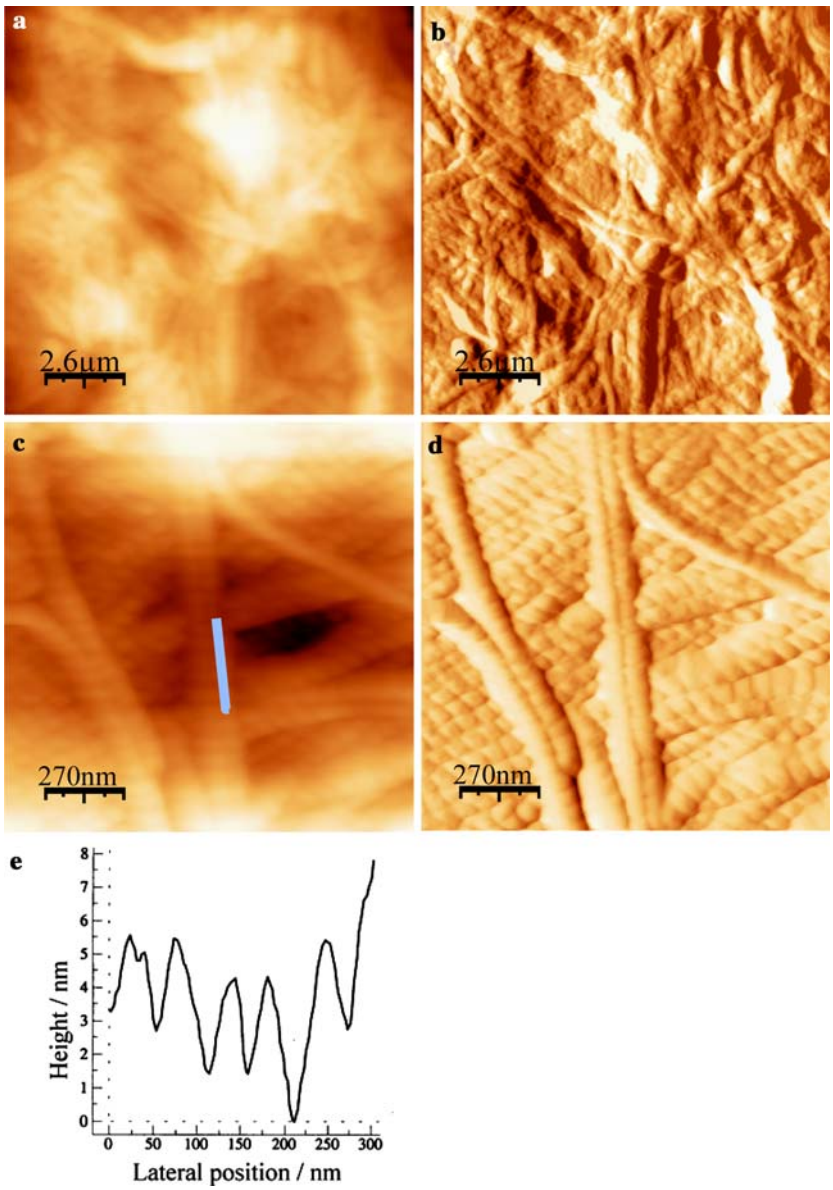

Fig. 1 AFM Height (**a**, **c**) and Deflection (**b**, **d**) images for the fibrosa layer (outflow) of the human aortic valve. The images (**a**, **b**) and (**c**,**d**) span the fields of (13 µm × 13 µm) and (1.3 µm × 1.3 µm), respectively. At higher magnification (**d**), the dense packing "in phase" of parallel collagen fibrils with the characteristic D-banding pattern is distinctly seen. The curve in (**e**) shows an axial profile of the collagen fibril taken along the marked line in the Height image in (**c**). The mean value of the D-period is (56 ± 6) nm

neighbouring grooves is equal to (65 ± 4) nm, which is typical for the I-type collagen.

Collagen fibrils can also form multilayered bands with dense packing of parallel thin fibrils, as shown in Fig. 3f. The dense packing is accompanied by the alignment "in phase" of the thin collagen fibrils, which has been previously observed for the aortic valve fibrosa layer. In Fig. 3f one can also observe small voids between bands of the densely packed fibrils. Fibrils with dense packing and aligned "in phase" reveal lower D-distance. Figure 3g shows an axial profile describing the surface topography of the densely packed "in phase" collagen fibril taken along the line shown in the Height image in Fig. 3e. The mean value of the D-period is equal to (54 ± 4) nm. For future analyses, it seems to be important to test mechanical properties of the composition of collagen fibrils with dense packing and aligned "in phase".

GA-stabilization procedure introduces considerable changes in the spatial organization of collagen fibrils as well as in the surface topography of the fibrils. Figure 4a and b show the Deflection images of the GA-treated porcine pericardium tissue. Fibrils are arranged in a disordered, tangled manner. At higher magnification, one can observe fibrils with strongly disturbed D-spacing pattern. The profile does not show the regular 60–70 nm D-periodicity. As shown in Fig. 4d, the distances between neighbouring grooves are considerably changed and their values are from about 150 nm to 270 nm. Periods longer than 70 nm are probably formed by coupling of two or three neighbouring ridges. The long spacing periods

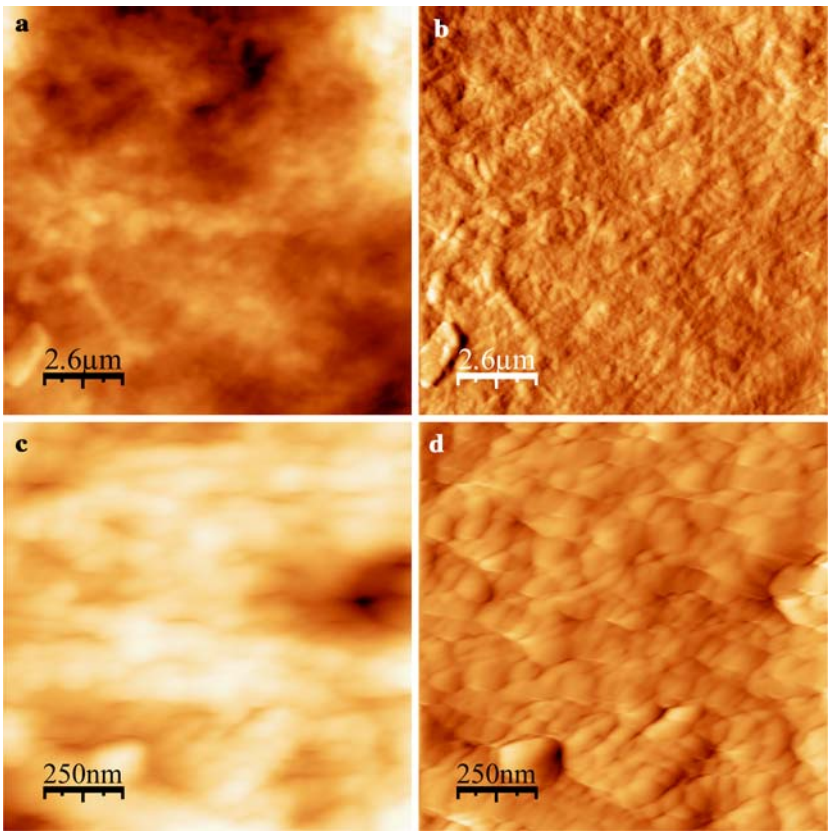

Fig. 2 AFM Height (**a**,**c**) and Deflection (**b**, **d**) images of the ventricularis layer (inflow surface) of the human aortic valve, at two different magnifications: (13 μm × 13 μm–images **a**,**b**) and (1.3 μm × 1.3 μm–images **c**, **d**). The ventricularis layer shows relatively smooth, amorphous surface. This layer is abundant in highly hydrated proteoglycans and elastin

correspond to the broadened regions of the fibril. These results are in good agreement with earlier findings obtained from the AFM investigations performed for chemically stabilized tissues [20].

It is widely accepted that GA-fixation introduces loss of compliance with the resultant stiffening of the tissue [1–3]. This effect can accrue through a combination of many factors that include: the protein cross-linking, changes in the surface topography of collagen fibrils, changes in collagen fibrils hydration. The presented AFM images show considerable changes in the surface topography of GA cross-linked collagen fibrils. It is expected that changes in the D-period result from a longitudinal shift as well as from an increase in the interaxial separation of tropocollagens within the fibril. Tropocollagen is a basic structural unit of the collagen fibril and posses a triple helical structure [27]. Increase in the interaxial distance between tropocollagen molecules was previously observed using Raman spectroscopy [26]. All these structural changes have an impact on the hydration shell surrounding tropocollagen. Greater distance between tropocollagens facilitates the growth of the hydration shell that can result in stiffening of the fibril. Interfibrilar cross-linking can correspond to the locking of dynamic structural rearrangement, which optimizes natural valve function. This in turn can induce delamination of the tissue.

Changes in the molecular conformation of GA cross-linked collagen may also contribute to the calcification of collagen fibrils. It can be hypothesized that the observed distortion in collagen structure could expose or produce new calcium nucleation sites and create internal spaces, which facilitate calcific crystal growth [28–30].

AFM imaging of the mineral deposits at their early stages, under slightly altered physiological conditions, could provide valuable information regarding calcification process.

Conclusions

The surface topography of human aortic valve in comparison to the native porcine pericardium and pericardial xenograft material was studied by the contact mode atomic force microscopy. The xenograft material was obtained by the glutaraldehyde-stabilization of the porcine pericardium tissue.

It has been found that pericardial collagen is not as compact as that of human aortic valve fibrosa. Pericardium has loosely arranged collagen fibrils. Small voids between collagen bundles are observed. Pericardial and aortic valve collagens reveal the regular transverse D-banding pattern with the mean value of the band intervals equal to (65 ± 4)

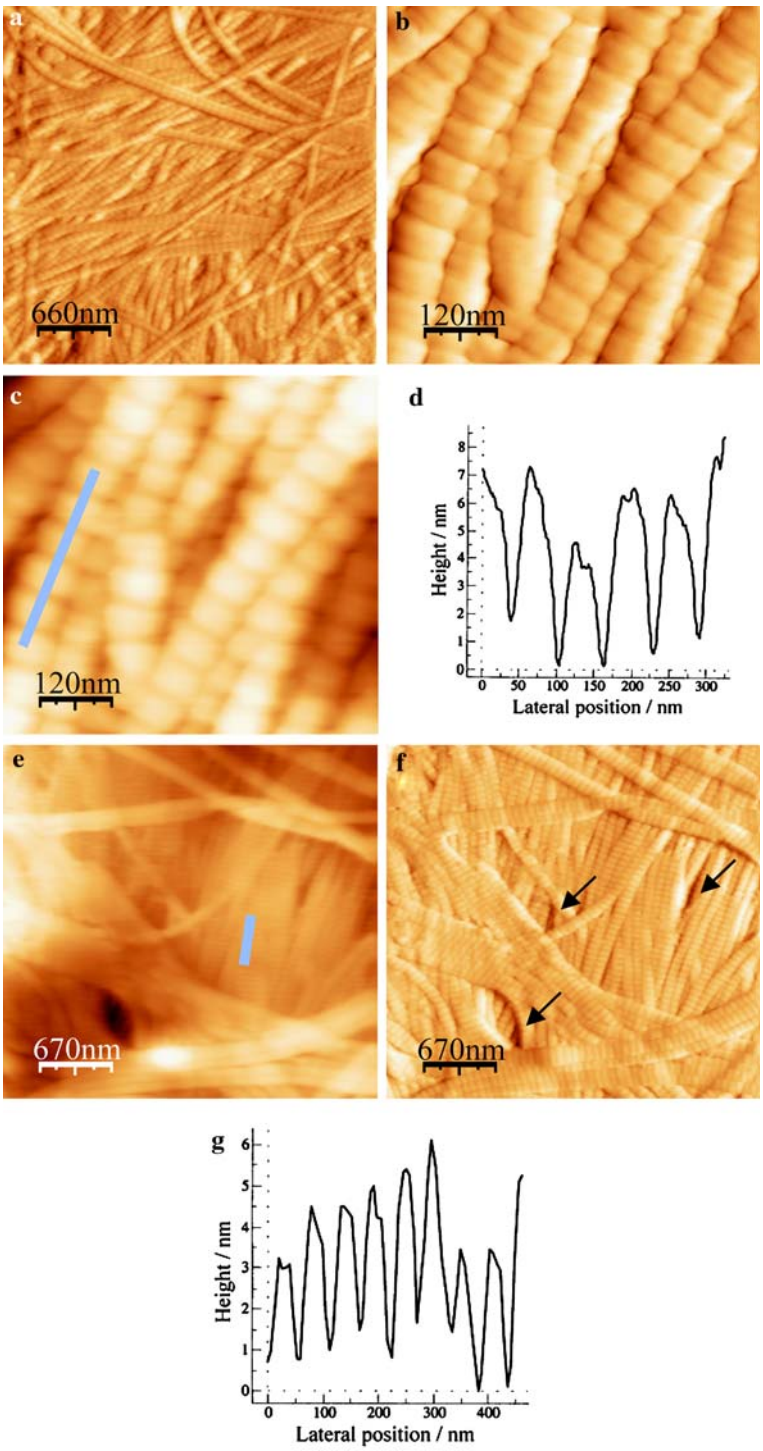

Fig. 3 AFM Deflection (**a**, **b**, **f**) and Height (**c**,**e**) images of the fibrosa layer of the native porcine pericardium tissue. Two different magnifications: (3.3 μm × 3.3 μm) image (**a**) and (620 nm × 620 nm) images (**b**, **c**). Collagen fibrils run in different directions. One can observe loosely arranged, multilayered fibril bands, which overlap one with each other. At higher magnification (**b**), the D-spacing pattern can be easily observed with the mean distance of (65 ± 4) nm. The curve in (**d**) shows an axial profile taken along the line seen in the Height image in (**c**). Height (**e**) and Deflection (**f**) images (3.3 μm × 3.3 μm) illustrate the dense packing, "in phase", of the parallel, thin collagen fibrils. An axial profile in (**g**) shows the surface topography of the densely packed and "in phase" collagen fibrils. The profile is taken along the straight line seen in the Height image in (**e**). The D-distance reaches the mean values of (54 ± 4) nm, which is lower in comparison to the value for loosely packed fibrils in (**b**). Arrows in the Deflection image (**f**) show small voids between bands of the densely packed fibrils

nm and (56 ± 6) nm, respectively. For both tissues, zones with multidirectional, stacked collagen fibrils and areas with fibrils packed regularly, densely and "in phase", are observed. It is supposed that different forms of fibril packing, i.e. dense or loose, have an impact on the collagen band interval. For the aortic valve, the majority of collagen fibrils show lower D-distance in comparison to the pericardium tissue. This can be due to higher compactness of the valvular collagen fibrils.

GA-fixation introduces considerable changes in the surface topography of collagen fibril as well as in the spatial organization of the tissue surface. The structural changes observed at the supramolecular level may contribute to the progressive damage and calcification of the

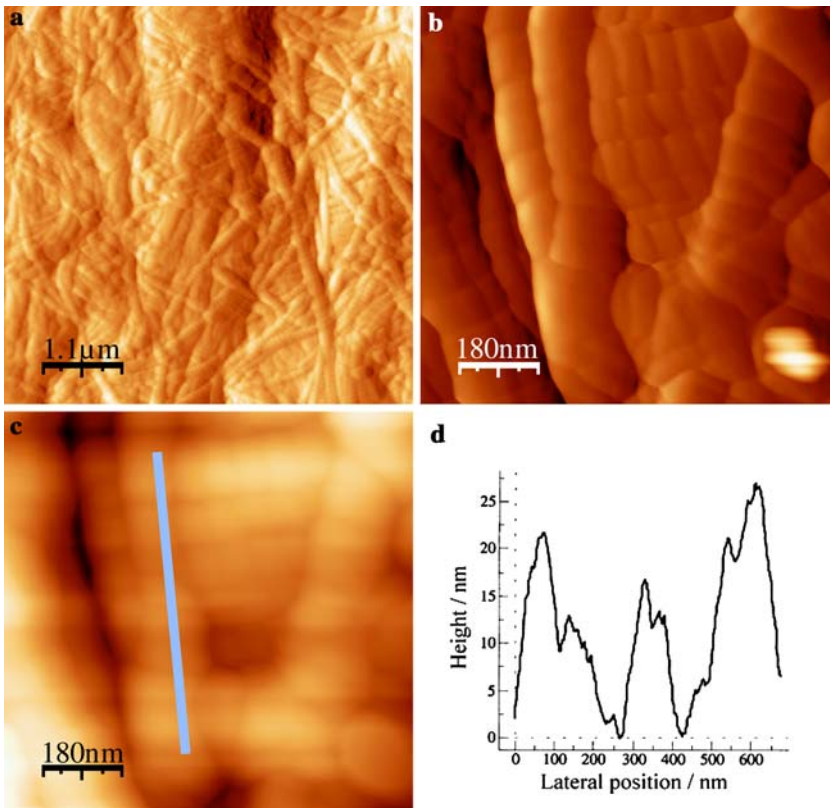

Fig. 4 AFM Deflection (**a**, **b**) and Height (**c**) images of the porcine pericardium tissue stabilized by glutaraldehyde (GA), at two different magnifications: (5.4 µm × 5.4 µm) and (890 nm × 890 nm). Stabilization with GA introduces considerable changes in the fibril arrangement on the tissue surface as well as in the surface topography of collagen fibrils. Fibrils run in disordered manner and, in comparison to the native pericardium or to the aortic valve, they do not form any more areas with regular, parallel alignment. The curve in (**d**) shows an axial profile of the fibril taken along the line seen in the Height image in (**c**). The D-spacing is strongly disturbed. Long periods consisting probably of two or three joint D-periods are observed

tissue. Loss of compliance of the GA-stabilized tissue can correspond to the changes in the internal structure and hydration of the fibrils leading to their stiffening. Changes in the internal fibril structure have been observed by AFM as the strong disturbance in the D-banding pattern.

Our results demonstrate that the AFM technique is a powerful tool for nano-scale structural characterization of natural heart valves and bioprosthetic heart valve material under slightly altered physiological conditions. Such investigations can be also essential to make improvement in the technology of bioprosthetic valves.

Acknowledgements The authors wish to thank Dr. J. Nozynski from the Department of Histology and Embryology of the Medical University of Silesia, Poland, for the preparation of human aortic valve and helpful discussion. This work was supported by the State Committee for Scientific Research (KBN, Poland) under Project No. NN-2-365/05.

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