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Fibrillin-rich microfibrils: an X-ray diffraction study of the fundamental axial periodicity

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Abstract Microfibrils are ubiquitous matrix polymers which are thought to provide elastic properties in all extracellular matrix structures. The major component of the elastic microfibrils is the protein fibrillin; its molecular structure is unknown. In electron microscopy, microfibrils appear as beaded structures exhibiting a variable periodicity, indicating that they may be elastomeric. The X-ray diffraction of fibrillin-rich microfibrils in the form of zonular filaments from bovine eyes exhibits meridional diffraction peaks indexing on a fundamental periodicity of 55 nm in the relaxed state. The application of a 40% extension produced a lengthening of the periodicity by 3% as judged by alteration of the D spacing of the principal peaks. This effect was shown to be reversible. Changes in the periodicity of the meridional reflections indicate changes in the fundamental structure of the microfilaments, but cannot account for all long range elastomeric properties of fibrillin-containing microfibrils.

Key words: Fibrillin; Microfibril; X-ray small angle scattering; Biomechanics

1. Introduction

A fibrillar network in the extracellular matrix (ECM) is formed from elastic microfibrils which are widely distributed in skin, tendon, muscle, cornea, vasculature, cartilage and at sites of epiphyseal growth [1]. In evolutionary terms, microfibrils may be the most fundamental elastic components of the ECM, and may therefore be of central importance in providing long-range elastic recoil to connective tissues. At present we have little evidence on the molecular structure and mechanisms of extensibility in microfibrils.

Isolated microfibrils are 10–14 nm wide beaded structures exhibiting a variable axial periodicity ranging from 33 to 165 nm [2]. This 400% variation in bead periodicity indicates that microfibrils may be highly elastomeric. To date only a tentative structural model has been proposed to account for this property [3]. Following isolation, their structure can be visualised by rotary shadowing or scanning transmission electron microscopy (STEM) analysis [2,4]. Strings with more than sixty beads have been observed in isolated microfibrils. The strings of beads have been observed to be highly flexible. The exact composition of the microfilaments in vivo is undefined, but the major component of the elastic microfibrils is known to be the protein fibrillin; a multidomain structure, it contains 47 epidermal growth factor-like domains which have calcium binding potential [5,6]. Its molecular arrangement is undefined at present. Fibrillin is believed to contribute both to the beads and to the filamentous strands linking the beads [7]. There is mounting evidence that although fibrillin is the major protein in microfibrils, other macromolecules must also be present [4,8,9].

The periodontal ligament and zonular fibres in the eye are examples of tissues containing elastic microfibrils but no detectable amorphous elastin [10]. X-ray diffraction studies of fibrillin in such tissues can provide information on molecular structure, packing and behaviour on extension that may explain the unusual biomechanical behaviour of microfibrils. The use of synchrotron radiation with large sample-to-detec-

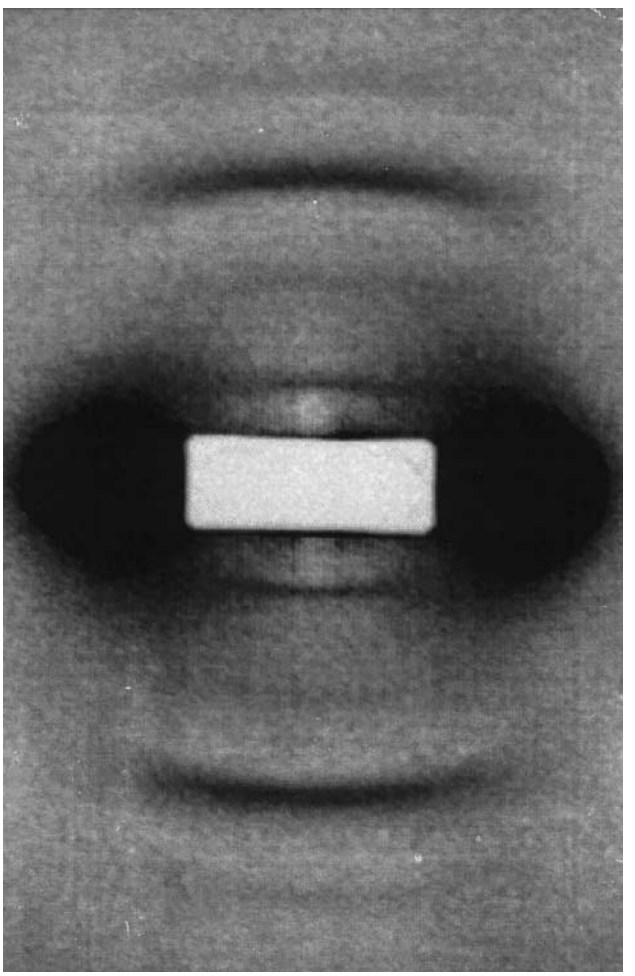

Fig. 1. X-ray diffraction image of fibrillin-rich zonular filaments. Data recorded on beamline 2.1 CLRC Daresbury. Camera length, 6.25 m. The predominant 1st and 3rd orders can be seen.

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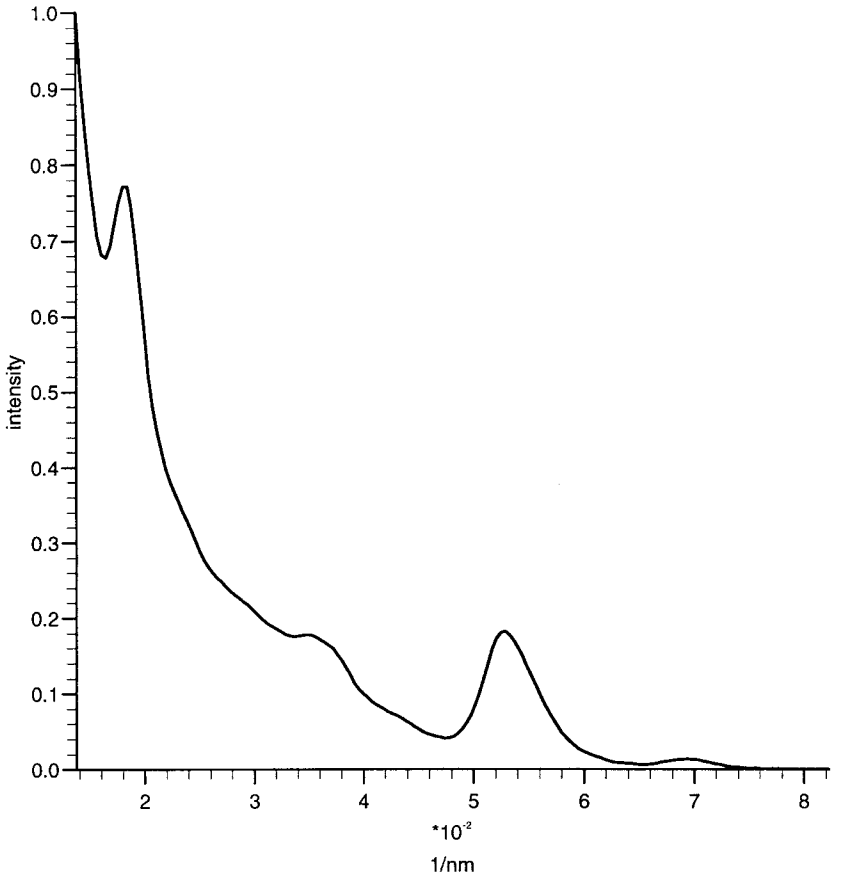

Fig. 2. Calibrated plot of the meridional peak intensity obtained using a 6.25 m camera, the 3rd order of diffraction being predominant at a reciprocal spacing of $5.3.\times10^{-2}$ nm^{-1}.

tor distances allows the study of weakly diffracting long range periodicities in biological structures.

2. Materials and methods

Zonular fibres from bovine eyes are relatively large (5 mm long in situ) and contain microfibrils orientated along their length. They therefore present an good system on which to study the structure and extensibility of fibrillin. We have established techniques for isolating the thin, aligned layer of zonular fibres intact from bovine eyes. Bovine eyes were obtained from a local abattoir from Friesian cross cows approx. 12–18 months old, within 24 h post mortem. Dissection through the posterior chamber of the eye yielded a preparation of the complete ciliary body and lens adhering to vitreous humour. Following enucleation of the lens, thin aluminium strips with internal rectangular apertures (typically 3×5 mm) were glued with cyanoacyrylate to the lens capsule at one end and the ciliary body at the other, such that the zonular filaments were positioned in the aperture and aligned parallel to its long axis. Careful dissection then released the zonular filament preparation mounted to the aluminium frame, together with some adhering vitreous humour. The vitreous humour was released by incubation with a 1 mg/ml solution of hyaluronidase (Sigma) for 8 hours, using 2 ml per sample. The preparation was washed copiously with phosphate buffered saline to remove loosely associated molecules.

Although the zonular filaments are one of the largest fibrillin aggregates to be found, they are nevertheless very thin (circa 50 μm) and required high X-ray flux in order to obtain a suitable signal to noise ratio. Beamline 2.1 at the CLRC Daresbury laboratory provided a suitable small beam of intense X-ray flux combined with a suitable low angle camera geometries. The sample was mounted between two thin sheets of mica that were sealed together with silicon grease and the sample bathed in phosphate buffered saline pH 7.5. The alumi-

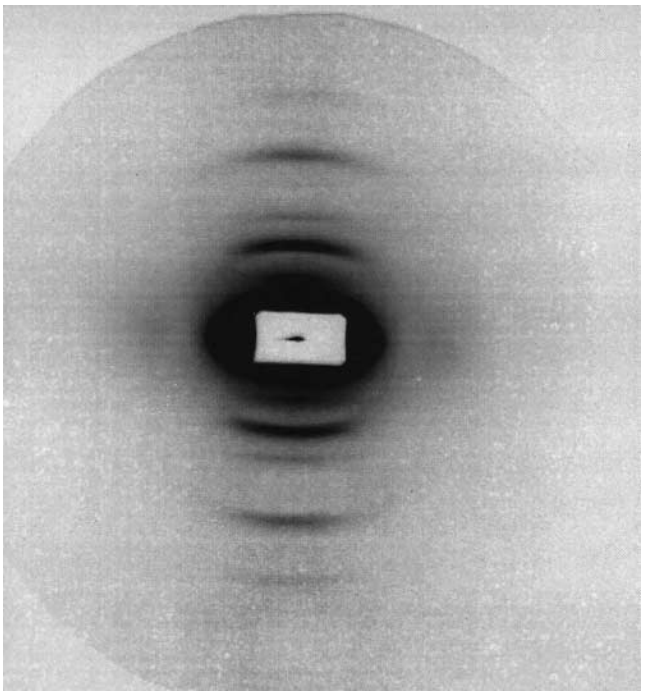

Fig. 3. X-ray diffraction image of fibrillin-rich zonular filaments. Data recorded on beamline 2.1 CLRC Daresbury. Camera length, 3.25 m. The 2nd–8th orders can be seen, with the 3rd and 6th orders having the strongest intensity.

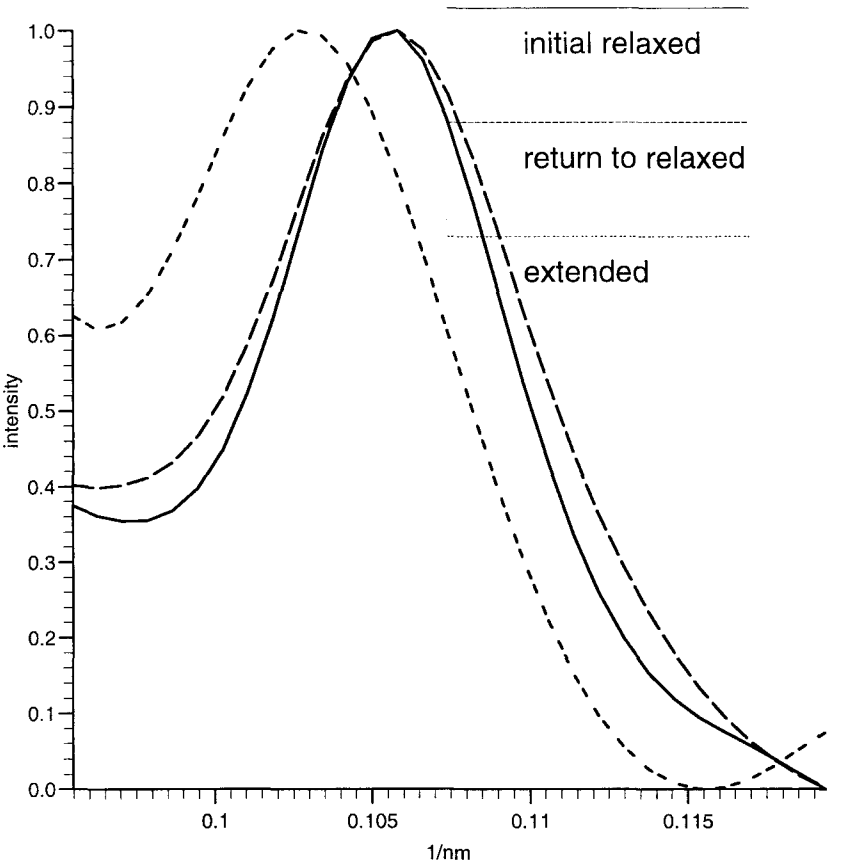

Fig. 4. Movement of the 6th meridional peak intensity. The plots show the intensity distribution along the meridian for initial relaxed (——), extended (– – – –), and returned to relaxed (— —) sample lengths. The changes in the position of the reflections is toward longer axial periodicity with extension (see text for detail).

nium frame facilitated both a suitable aperture for the X-ray diffraction beam and also a means of holding the sample. In order to apply extension, the long sides of the frame were cut after mounting its ends in a clamps, which were then moved a set distance. Data was collected for samples in both relaxed (unextended) and extended (40% increase in sample length) states. Samples were also returned to original rest lengths in order to examine the reversibility of the elastic properties of the system.

The sample to detector distances used were 3.25 m and 6.25 m (beamline 2.1) with an X-ray beam spot size at the sample of 200×500 μm defined by slits. The wavelength of X-rays used was 0.1488 nm. Data was collected on a 2-dimensional gas filled detector developed at CLRC Daresbury. Suitable blank exposures of the empty cell and a detector response were taken. A sample of wet rat tail tendon was used for calibration and comparison of diffraction strength. The exposure time of each fibrillin sample was 15 min (rat tail tendon 2 min).

TEM studies of samples used in X-ray diffraction experiments conducted as follows. Samples were fixed for 2 hours in 2.5% gluteraldehyde postfixed in 1% OsO_4 and embedded in EPON. Ultrathin sections were made and stained with uranyl acetate and lead citrate. Samples were examined in a Philips EM201 at an accelerating voltage of 60 kV images taken at 20000× magnification.

2.1. Data analysis

X-ray diffraction 2-D images were calibrated using programs written to utilise the 1/67 nm^{-1} meridional axis spacing of rat tail tendon Bragg peaks. All images were corrected for the detector response and the empty cell was subtracted from each. Data pixels were converted from 2-D detector data to radial data profiles (Reciprocal film space co-ordinate vs. angle where the equator is 0 degrees) in order to facilitate integration around arced Bragg diffraction peaks. The diffraction image from a 6.25 m camera length can be seen in Fig. 1. Data correction procedures were similar to those used in [11]. The 2-D radial distribution profiles were integrated using boundaries that included all visible meridional Bragg intensity; this resulted in single line plots used to examine any changes in meridional intensity position.

3. Results

3.1. X-ray diffraction

The X-ray diffraction patterns exhibited a weakly diffracting series of meridional Bragg peaks. The background diffuse scatter is believed to derive in part from the sample itself, indicating a degree of static disorder in the sample. The application of tension to the sample caused the shape of the diffuse scatter to become anisotropic, also indicating that it derived from the sample (Fig. 1). X-ray diffraction data were obtained from five different preparations of zonular filaments; each produced very similar diffraction patterns.

The major Bragg peaks observed with the 3.25 m camera geometry correspond to periodicities of 9.46 and 18.86 nm, respectively. These can be indexed on a fundamental periodicity of 56.56 nm. The data obtained from the 6.25 m camera geometry indicates the presence of further peaks with longer range order, indexing on a fundamental periodicity of 56.48 nm. The small discrepancy between measurements of fundamental periodicity is due to experimental error of peak measurement.

The first four orders of diffraction can be seen in Figs. 1 and 2. The first order is close to the backstop, the second and

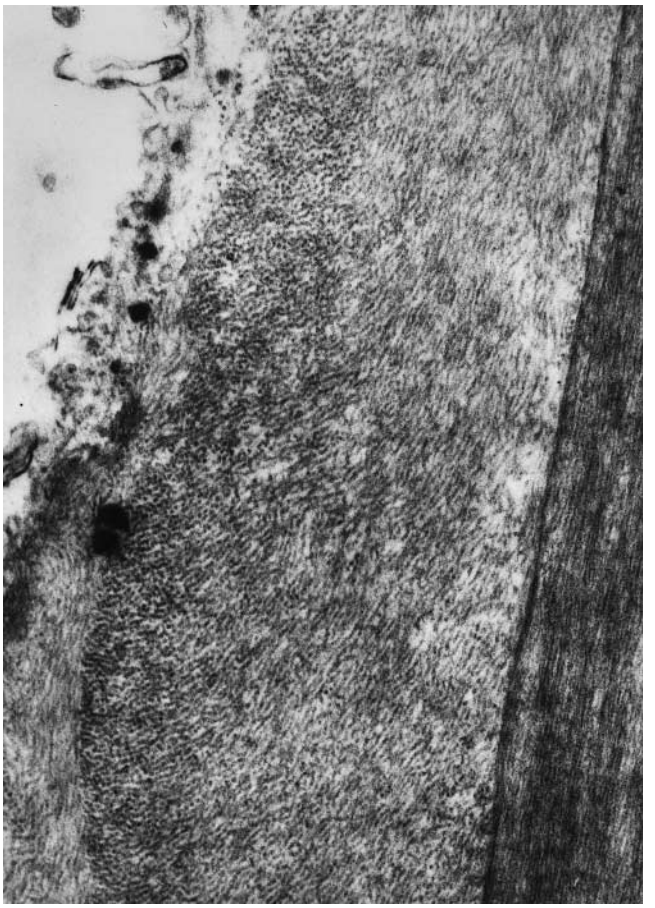

Fig. 5. TEM of ciliary zonule filaments from bovine eyes, following exposure to X-ray beam. Zonules were fixed and stained as described in Section 2. Abundant microfibrils with an approximate diameter of 10–12 nm are clearly apparent. (Original magnification ×20 000).

fourth orders can be discerned above the background. Fig. 3 shows a diffraction image collected using the 3.25 m camera length. Extension of the tissue by 40% caused the relative position of the Bragg peaks to index on a longer periodicity of 58.42 nm, as judged by changes in the position of the sixth order of diffraction (see Fig. 4).

3.2. TEM

The samples used in X-ray diffraction were examined by transmission electron microscopy. All samples revealed a high density of microfibrils (see Fig. 5). Regions of the zonules studied by TEM indicated that the samples used were not contaminated with basement membrane matrix. It is difficult to discern the beaded nature of filaments in intact tissues; the diameter of the fibrils corresponds is approx. 15 nm, which corresponds to the characteristic size of fibrillin-rich microfibrils.

4. Discussion

The small angle X-ray diffraction data presented here contains important elements of information relating to microfibrillar structure:

The microfibrils have an ordered structure that contains periodic features in the relaxed state. The periodicities correspond to structural features of electron density above the average axial value. The principal peaks correspond to spacings at 56.56, 18.86 and 9.46 nm. The first-order meridional diffraction peak (as reported from the periodicity observed in TEM studies [3]) could not be observed using the 3.25 m camera due to the camera geometry, but a peak was clearly observable with the 6.25 m camera.

The presence of diffraction peaks indicates that the axial structure of microfibrils contains features within the bead-interbead periodicity. This may result from the arrangement of EGF type domains within the bead interbead regions [12] or the periodic interaction of fibrillin with other proteins such as microfibril associated glycoprotein [9]. Antibody labelling studies have indicated that the labelling of the beaded region is asymmetric. This suggests that there may be fundamental structural differences across the bead region that may give electron density contrast, resulting in X-ray Bragg peak intensity at certain periodicities [5]. The analysis of the diffraction pattern described here indicates that the Bragg peaks index as the 3rd and 6th orders of an approx. 56 nm periodicity in the relaxed state. The strength of the 3rd and 6th orders of diffraction indicate that there is structural variation within the microfibrils, or that there is specific alignment or staggering of adjacent microfibrils that gives rise to electron density contrast at periodicities one third of the axial unit cell length.

The stretching of the zonular filament alters the periodicity of the observed Bragg peaks to index on a one-dimensional lattice with a longer periodicity. The elastic properties of microfibrils are central to their function. The physical basis of these properties are not well understood. If changes were to occur to the fundamental periodicity on stretching; it would become increasingly difficult to see the first order of diffraction since the diffraction peak would subtend a smaller diffraction angle. Measurements can be measured more accurately and conveniently at higher index orders of the meridional spacing. Due to the inherent breadth of the Bragg reflections resulting from incoherence in the microfibrillar structures, the diffraction intensities become weak after 8 orders of diffraction. The 6th order was chosen to measure the movement of the diffraction peaks since it is relatively strong.

Extension of the tissue showed a 3% change in the axial repeat of fibrillin microfibrils (see Fig. 4); the tissue was returned to the relaxed state and a diffraction image obtained with identical periodicity to the original relaxed state. This indicates that the zonular filament extension relates to reversible changes in microfilament axial periodicity. The changes in the periodicity are however very small in comparison to the extension applied. This indicates that the changes in the fundamental bead-interbead periodicity cannot explain the elastic behaviour of fibrillin-rich tissues.

5. Conclusion

The X-ray diffraction study presented here indicates that the fibrillin periodicity can be observed in intact hydrated tissue samples and the lattice corresponding to the axial repeat is extended to a small extend on stretching. This is the first time that X-ray diffraction images have been obtained for fibrillin microfibrils and also the first proof that the fundamental periodicity of fibrillin is present in the native hydrated state. The extension of the lattice spacing appears to be small compared to the extension of the whole tissue and therefore

we suggest that bead interbead spacing changes cannot account for all the macroscopic elastic properties of tissues such as zonular filaments. The X-ray diffraction data presented here is of importance since further models of microfilament structure and elastic behaviour must be able to account for the diffraction intensity and the changes in diffraction profile on stretching.

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