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Collagen orientation by X-ray pole figures and mechanical properties of media carotid wall

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Many natural collagen containing materials are highly extensible composites and their mechanical behaviour will depend on the amount of collagen fibres present, the mechanical properties of the fibres and their distribution and orientation. A characteristic feature of these materials is that the fibrous collagen network can change orientation during stretching and hence the mechanical response of the tissues is a non-linear function of stress. In order to study the effect of fibre orientation, samples of carotid artery have been prestrained by given amounts in the hydrated state and then allowed to dry. The mean orientation of the fibres has been derived using pole figures obtained from X-ray diffraction measurements and Young's modulus has been calculated in the direction of fibre reorientation and compared with the experimental measurements. The results obtained indicate that X-ray diffraction techniques can be used for the study of the mechanical properties of extensible fibrous materials.

1. Introduction

Most soft connective tissues such as skin, cartilage, tendon and blood vessels can be considered as flexible fibrous composites in which relatively inextensible high modulus fibres (collagen) are embedded in a matrix of elastin, polysaccharides and water. In common with artificial composites, the mechanical properties of such materials will be strongly dependent on the orientation of the fibrous phase. In order to meet specific mechanical requirements, several fibre morphologies are found in biological materials: parallel fibres in tendon [1], crossed-fibrillar arrays in the membranes of the sea anemone [2], cross-helical arrangements in fish skin [3, 4] and random distributions, both planar and three-dimensional, as in skin [5, 6].

The latter arrangement seems to be also present in unstretched elastic blood vessels in general. The media wall of these vessels consists of concentric lamellae of elastin surrounded by smooth muscle cells and an intricate network of collagen fibres [7, 8]. In the hydrated tissue the collagen fibres have a certain degree of freedom which allows them to orient in the direction of an applied stress. This effect produces a non-linear stress–strain behaviour characterized by an increase in Young's modulus with strain.

Many authors [7–13] have attempted qualitative and quantitative descriptions of this behaviour using a variety of physical and mathematical models, few of which are based on direct measurements of the orientation of the collagen fibres during stretching. In a recent work [14] we have shown that there is a correlation between collagen orientation, measured by X-ray diffraction techniques, and the uniaxial stress–strain curve of aortic media layer.

In the present paper, in order to explore the possibility of using quantitatively the information obtained on the orientation of the fibres, we have restricted ourselves to the study of dried tissues in which different degrees of orientation have been induced by prestretching the material in the wet

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state. In doing this we are treating the samples of dried carotid artery as a stiff fibre reinforced material with breaking strains of only a few per cent. This allows the use of well established theories for the description of the mechanical properties.

2. Theory

2.1. Mechanical properties

Using network analysis, it has been shown that the Young's modulus of a composite reinforced with continuous fibres depends on the properties of the fibres and matrix, their respective volume fractions and the orientation of the fibres [15]. The results obtained are also valid for discontinuous fibres provided that they are long enough for efficient load transfer between fibre and matrix to take place (this assumption has been made in this paper). If the wall of the carotid artery is considered as a composite with a three-dimensional network of fibres whose orientation with respect to the direction of stretching (z) is defined by the angles θ and ϕ (Fig. 1), the stiffness constants C_{ij} of the system will be a function of the moduli of the fibres and matrix and of the angular distribution of the fibres. Furthermore, if the material is considered transversely isotropic throughout the deformation along z*, it can be shown that the composite modulus E_z in the direction z will be given by

$$E_z = C_{33} + \frac{2C_{13}^2(C_{12} - C_{11})}{(C_{11}^2 - C_{12}^2)}. \quad (1)$$

The second term in Equation 1 is generally small compared to C_{33} and can be neglected as a first approximation. Introducing the angular dependence, Equation 1 becomes

$$E_z = C_{33} = E_f v_f \int_0^{2\pi} \int_0^{\pi/2} \cos^4\theta F(\theta, \phi)\, d\theta\, d\phi + (1 - v_f)E_m \quad (2)$$

where E_f and E_m are the Young's moduli of the fibres and the matrix, v_f is the volume fraction of fibres and $F(\theta, \phi)$ is an angular distribution function which varies with the degree of stretching along z. Because of the transverse isotropy, the integration with respect to ϕ can be carried out

$$E_z = E_f v_f 2\pi \int_0^{\pi/2} \cos^4\theta F(\theta)\, d\theta + (1 - v_f)E_m. \quad (3)$$

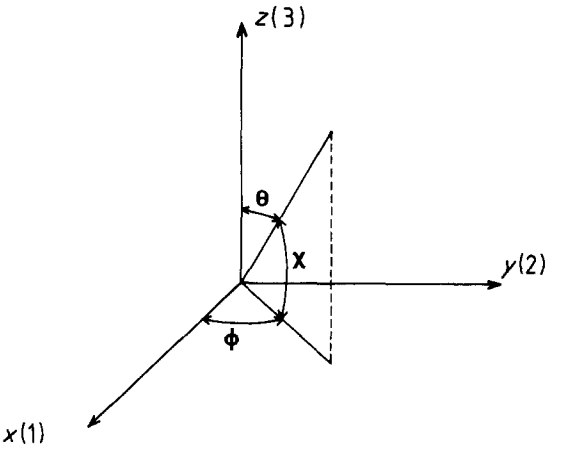

Figure 1 Co-ordinates reference system for mechanical and X-ray analysis. The stretch direction is z.

For the special case of a three-dimensional random distribution, the number of fibres between θ and $(\theta + d\theta)$ is proportional to the solid angle $\sin\theta\, d\theta\, d\phi$ and integrating Equation 2 gives

$$E_z = \tfrac{1}{5}E_f v_f + (1 - v_f)E_m. \quad (4)$$

2.2. X-ray diffraction

The X-ray diffraction techniques provide various methods for the determination of the direction and degree of orientation in oriented specimens. One of them uses pole figures i.e. stereographic projections showing the density of crystallographic poles of certain planes as a function of orientation [16]. If we construct a sphere whose centre is the centre of a crystal, a pole will mark the point of intersection between each perpendicular drawn from a crystal plane and the surface of the sphere. If the sphere has a radius "$r = I/d(h\,k\,l)$", then each pole figure is also the reciprocal lattice point of the crystal plane and the density distribution of such points corresponds to the distribution of poles over the sphere. If the crystallites are oriented at random, no systematic pattern will appear in the pole figures. With increasing degree of orientation the poles will be concentrated preferentially in some areas of the projection and the pole density, which is proportional to the intensity of the scattered X-rays, will provide a measure of the degree of orientation.

In the present investigation the angle between the fibre axis of collagen and the direction of stretching is θ (Fig. 1). The mean value of $\cos^4\theta$

*This assumption of transverse isotropy has been verified in a previous work [14].

for various degrees of orientation have been calculated directly from the intensity distribution function $I(\chi, \phi)$ of the meridional reflection used for the construction of the pole figures. The number of planes whose normals have co-latitude $\theta = 90° - \chi$, falling between θ and $(\theta + d\theta)$, is proportional to $I(\theta) \sin\theta d\theta$; the value of $\cos^4\theta$ averaged over all possible angles is given by

$$\langle\cos^4\rangle = \frac{\int_0^{2\pi}\int_0^{\pi/2} I(\theta, \phi) \sin\theta \cos^4\theta \, d\theta \, d\phi}{\int_0^{2\pi}\int_0^{\pi/2} I(\theta, \phi) \sin\theta \, d\theta \, d\phi} \quad (5)$$

where the intergrals are approximated by summation. The intensity distribution function, obtained from the X-ray diffraction patterns, represents a measure of the angular distribution $F(\theta, \phi)$ and thus Equation 5 can be used to estimate the integral in Equation 2.

3. Materials and methods

Samples of bovine carotid artery were obtained from nine-month old animals and segments of blood vessel 100 mm long were removed within 1 h of death and stored in a solution of 1% NaCl at − 30° C until tested. All experiments have been carried out within two weeks of obtaining the specimens. The artery segments were further divided into rings 20 mm high from which circumferential strips were cut for mechanical testing and X-ray analysis. In all specimens the adventitia was removed leaving only the media and intima layers. The strips used for testing were about 20 mm long, 5 mm wide and 1 to 2 mm thick in the fully hydrated condition. The specimens were circumferentially stretched by given amounts and left to dry in air for at least 24 h before being used. In order to make sure that the axial strain in the samples did not change significantly with drying, two very small pins (0.25 mm in diameter) were inserted in the strips to provide a reference length. After drying, the distance between the pins was measured and it was found that there were no appreciable changes in the stretching direction, whereas greater variation was observed in the transverse dimensions.

All the mechanical tests were carried out at room temperature in an Instron tensile testing machine. Strain gauges were glued in the centre of the strips for the determination of the Young's modulus. The X-ray diffraction analysis was carried out on a series of strips before mechanical testing, the area of specimen exposed to the X-ray beam being chosen in the centre in order to avoid, as far as possible, the regions which may have been affected by end effects due to clamping.

The 0.29 mm reflection corresponding to the meridional periodicity of the triple helical structure of the collagen molecule was used for the analysis. The pole figure measurements were obtained using a Philips single-crystal diffractometer automatically controlled by a computer using filtered MoKα radiation. The carotid sample was fixed to the holder with the direction of deformation parallel to the axis $\theta = 0$ of the diffractometer; in this way the angular co-ordinates of the spherical projection of the pole figures were deduced directly from the position of the ϕ and χ circles.

The scanning of a complete hemisphere was obtained varying ϕ from 0° to 360° at 60° intervals and χ from 0° to 90° at 10° intervals. Scanning ϕ at 60° intervals only will mean a high degree of uncertainty in drawing the contour lines of the pole figures but this was considered acceptable as a first approximation. The intensity of the background as a function of ϕ and χ was determined by means of twenty scans around $\theta = 7.0°$ corresponding to the 0.29 nm reflection; the intensities above background were taken from the intensity profiles for successive given values of ϕ and χ. The small wall thickness of the samples made it unnecessary to apply a correction for X-ray absorption.

The determination of the volume fractions of collagen and elastin in the specimens was done biochemically. Samples from the centre of each strip were fully dried in a dessicator to a constant weight and hydrolysed in boiling 6 N HCl for 24 h. The acid was removed by evaporation *in vacuo* and the hydrolysates were analysed by ion-exchange chromatography using a JCL-5AH analyser taking care to have a good resolution of 4–OH proline, isodesmosine and desmosine [17]. Collagen and elastin. The value of collagen was corrected for the isodesmosine + desmosine contents, respectively, assuming that hydroxyproline represents 1.78% of elastin. The value of collagen was corrected for the amount of hydroxyproline found in elastin. The weight fractions obtained were taken to be equal to the volume fractions of the two components, since the difference in density between collagen and elastin is minimal.

4. Results and discussion

The recordings of the 0.29 nm reflection pole figures for the carotid artery were taken on specimens prestrained circumferentially by the following amounts, ϵ (%): 0, 20, 40, 60, 80. Figs 2 and 3 show typical pole figures obtained from samples prestrained by 40% and 80%. The contour lines have been chosen arbitrarily to give five equally spaced contours between the maximum and minimum intensities. The main feature illustrated by Figs 2 and 3 is the clustering of the crystallographic poles in the direction of stretching. The equitorial planes of the pole figures are perpendicular to the direction of stretching. The high intensity peak in the centre of the figures indicates that the stress axis is the main direction of orientation of the collagen fibre.

The results obtained show that there is a great number of orientable fibres which align along the direction of stretching even for small uniaxial deformations: the values of $\langle\cos^4\theta\rangle$ derived from the X-ray analysis of the prestretched carotid samples have been plotted in Fig. 4 as a function of ϵ. The standard deviation on the results is of the order of ± 0.02 for stretched samples attributable to the structural inhomogeneity of the tissue which depends on their biological nature. However, it must be noted that for $\epsilon = 0\%$ there is not sufficient contrast for an accurate measure of the intensity distribution. Therefore in this case the calculated value of $\langle\cos^4\theta\rangle$ greater than 0.2 must not be considered as clear evidence of a preferential orientation of the collagen fibres. Fig. 4 shows that orientation increases rapidly between 0 and 60% and levels off thereafter, tending to the limit value given by $\langle\cos^4\theta\rangle = 1$ for full orientation.

In Fig. 5 the values of E_z (experimental) have been plotted as a function of $\langle\cos^4\theta\rangle$ calculated from the X-ray diffraction data. There seems to be a good quantitative correlation between the

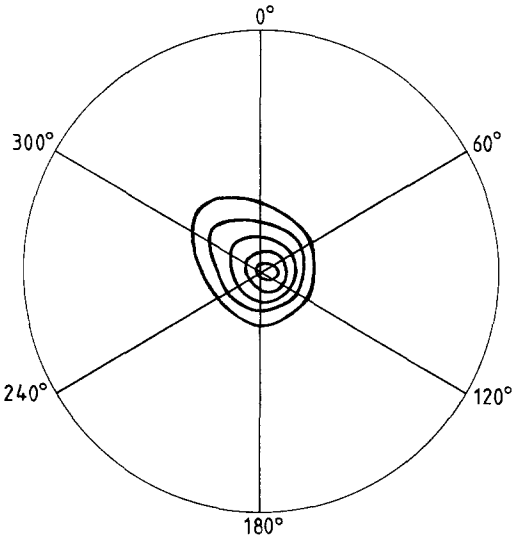

Figure 3 Pole figure for a blood vessel sample prestretched by 80% in the z-direction.

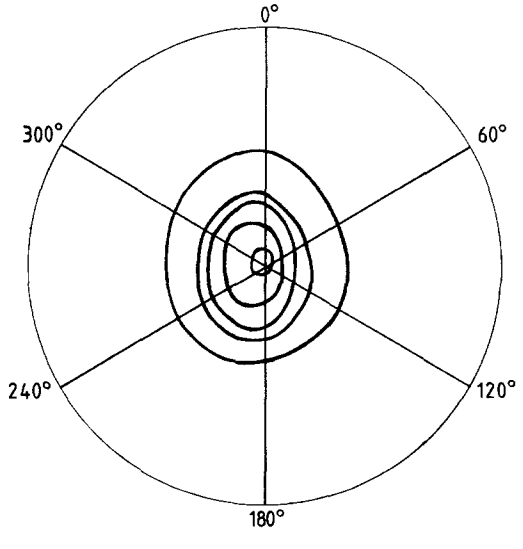

Figure 2 Pole figure for blood vessel sample prestretched by 40% in the z-direction. The z-axis is normal to the paper through the centre.

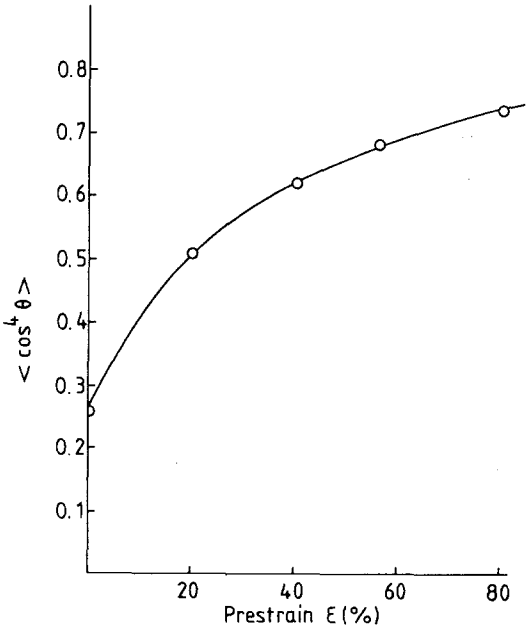

Figure 4 Experimental values of $\langle\cos^4\theta\rangle$ as a function of prestrain ϵ.

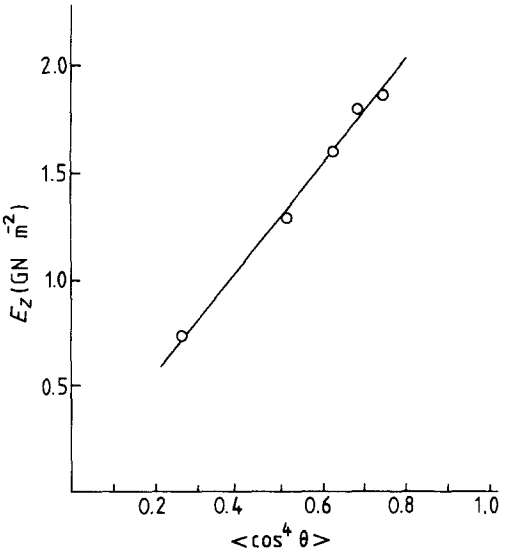

Figure 5 Experimental values of Young's modulus E_z as a function of measured values of $\langle\cos^4\theta\rangle$.

increase in orientation with stretching and the increase in modulus.

In order to make a quantitative comparison between theory and experiment, Equation 3 has been used where $\int_0^{\pi/2} \cos^4\theta F(\theta)\, d\theta$ has been estimated from $\langle\cos^4\theta\rangle$ reported in Fig. 4.

The Young's modulus, E_f, of dry collagen has been taken as 10^{10} N m^{-2} [18] whereas the modulus of dry elastin, E_m, has been measured directly on decollagenated samples; a mean value of 2×10^8 N m^{-2} being obtained.

From the results of the biochemical analysis a value of 0.27 has been found for the volume fraction of collagen.

The comparison between calculated and measured values of E_z as a function of collagen orientation are reported in Table I. The agreement between the two sets of values is good in spite of the approximations made.

The value of E_z for $\epsilon = 0\%$ suggests that the distribution of collagen fibres in the unstretched samples is a random three-dimensional distribution. In fact for a purely two-dimensional distribution it can be shown that E_z is given by

$$E_z = \tfrac{3}{8}E_f v_f + (1 - v_f)E_m \qquad (6)$$

which gives a value of 1.16×10^9 N m^{-2}, whereas for a three-dimensional distribution, using Equation 4, E_z takes a value of 0.65×10^9 N m^{-2} in agreement with the experimental result of 0.70×10^9 N m^{-2}. This result seems in contrast with the slight preferential orientation of the collagen fibres along the circumferential direction observed for the unstretched samples. However, the poor accuracy of the pole figures for these samples does not allow us to affirm with certainty the existence of an appreciable collagen orientation which, on the other hand, might be due to shrinkage during drying.

TABLE I Comparisons between calculated and experimental values of Young's modulus as a function of prestrain, ϵ, in dry carotid artery

Prestrain (%)	E_z (calculated) ($\times 10^9$ N m^{-2})	E_z (measured) ($\times 10^9$ N m^{-2})
0	0.84	0.70
20	1.53	1.10
40	1.83	1.50
60	2.00	1.80
80	2.10	1.95

The results which have been reported confirm that the use of pole figures derived from X-ray diffraction analysis can be used for the description of the fibre distribution and mechanical properties of biological tissues. Although this paper is limited to the study of dry tissues, this work suggests that similar methods could be used for the study of the stress–strain behaviour of hydrated biological materials when fibre reorientation during *in vivo* stretching is likely to be a very important factor for the mechanical response to applied loads.

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