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Structure of reconstituted collagen hollow fibre membranes

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Small- and wide-angle X-ray scattering (SAXS and WAXS) methods were employed to study the structure of reconstituted collagen hollow fibre membranes and the changes that ensue upon entry of water. The tails of the SAXS curves were analysed and were shown to obey Porod's Law. WAXS and water absorption measurements as a function of relative humidity were combined with density measurements to determine the relative volume fractions of water in the "free" and "bound" states. Treating the hollow fibre as a two phase system and employing Porod's Law, average length parameters transverse to the fibre axis were extracted for the collagen fibrils and the water filled pores. All this information was synthesized to yield a model of the structural changes in the hollow fibre caused by water. Implications of such a model for qualitative and quantitative prediction of changes in properties were studied.

1. Introduction

Collagen is the most abundant protein in animals. It is an important component of biological membranes and functions as a supporting structure in connective tissues. The basic unit of collagen commonly called "tropocollagen" is an asymmetric rod-like molecule about 2800 Å long and 10 Å diameter with a molecular weight of about 300 000. The molecule is composed of three similar but non-identical peptide chains wound into a triple helix [1]. Tropocollagen (TC) molecules can further aggregate in an orderly fashion to form higher level structures, commonly called the "protofibril" and the "fibril". The protofibril is essentially an end to end aggregate (polymeric chain) of TC molecules. Several such protofibrils can aggregate lateraly to form fibrils with diameters up to several microns [2].

Small non-helical appendages called "telopeptides" on collagen are responsible for many of its biological properties [3]. Techniques are now available for isolating and purifying large amounts of animal collagen at relatively low cost and useful membranes can be fabricated from such purified collagen. These techniques rely on the ability of proteolytic enzymes to remove the telopeptides [1, 4] resulting in collagen soluble in acidic solutions, which can then be precipitated by raising the ionic strength or pH and cross-linked by ultraviolet irradiation [5]. Removal of telopeptides also eliminates immunological reaction with antibodies to native collagen [6].

Such enzyme-treated collagen can be restructured into a variety of forms (membranes, hollow fibres, gels, etc.) and has excellent prospects as a candidate for biomedical applications. The projected uses for this material are discussed in detail elsewhere [7]. The purpose of the present research was to determine the microstructure of hollow fibre membranes fabricated from enzyme solubilized collagen as it relates to the intended use of these membranes in artificial kidney dialysis.

2. Materials and methods

Hollow fibres about 15 to 20 μm thick (dry dimensions) fabricated from reconstituted collagen are the material used in the present investigation. A brief description of the method of manufacture follows. The collagen was solubilized from calfskin with proctase and purified by repeated reconstitution. Hollow fibres were prepared by extruding a 2 to 3% solution of enzyme solubilized collagen into a coagulation bath consisting of an aqueous solution of saturated NaCl. The hollow fibres were irradiated with ultra-violet light to cross-

link them after coagulation, then neutralized with NaOH, washed and dried. Hollow fibres in the finished form were supplied by Dr Teruo Miyata of the Japan Leather Company.

Collagen is highly hydrophilic and consequently the hollow fibres when placed in water swell to dimensions of about 65 μm in thickness and 650 to 700 μm i.d. This large amount of water uptake causes dramatic changes in the structure and properties of the membranes. Small- and wide-angle X-ray diffraction coupled with other techniques were used to study changes in the structure of these membranes caused by water.

3. Experimental results

3.1. Water absorption isotherms

The weight of water absorbed by the hollow fibres as a function of relative humidity was determined using a Cahn electrobalance. The required relative humidity conditions were established by placing the samples above different glycerine–water solutions. The absorption isotherm at room temperature (23°C) is shown in Fig. 1 and belongs to class II of the B.E.T. (Braunauer, Emmett and Teller) theory [8]. Here the adsorbent (collagen)–water molecule attraction is stronger than the interaction between the water molecules themselves. The absorption isotherm is almost identical to those obtained by other investigators using collagen from native tendon [9]. The dotted line in Fig. 1 beyond 90% relative humidity corresponds to water absorption from the vapour phase up to a relative humidity of 100%, while the solid point refers to the amount of water absorbed by the hollow fibre when it is placed in contact with liquid water. The amount of water absorbed in the liquid phase is several times larger than the water absorbed in water vapour.* Since the membranes will function in an aqueous environment during hemodialysis, the water absorbed while in contact with liquid water is of primary importance.

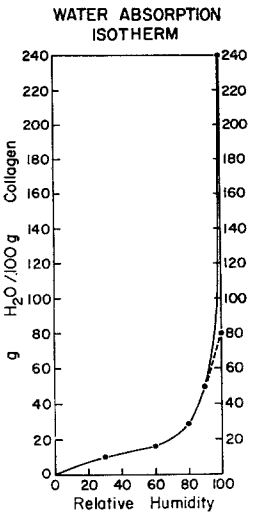

Figure 1 Water absorption isotherm for reconstituted collagen hollow fibre at room temperature.

3.2. Volume fraction water in wet hollow fibres

The density of the wet hollow fibre was measured using a pycnometer filled with water [10]. This method required no squeezing of the hollow fibres to remove excess water and was found to give more reproducible values than an alternative density gradient column method [11]. The measured average density was 1.0575 g cm^{-3}. Considering the wet hollow fibre as a two phase mixture (collagen and water) we can write for the volume fraction water (v_{H_2O}),

$$v_{H_2O} = \frac{1}{\rho_{H_2O}} [\rho_{HF} - v_c \rho_c] \quad (1)$$

where ρ_{HF}, ρ_c and ρ_{H_2O} are the densities of the wet hollow fibre, dry collagen and water respectively and v_c is the volume fraction of collagen. But since $v_c\rho_c = V_c\rho_c/V_{HF}$ where V_c and V_{HF} are the volumes of collagen and the wet hollow fibre, we can find $v_c\rho_c$ by taking the ratio of the weight of dry collagen to the volume of the wet hollow fibre. The volume of the wet hollow fibre was determined by measuring its dimensions under a microscope. The weight of dry collagen was determined by using the Cahn electrobalance. Using this information the volume fraction water is 0.85 ± 0.02.

An alternative way to compute the volume fraction water in wet hollow fibres is to measure directly the dry and wet volumes with the microscope. Although the uncertainty in this method is greater given the relatively large uncertainty in the measured dry volume, the volume fraction water measured in this way is 0.85 ± 0.04 which is in excellent agreement with the value quoted above.

A discussion of how the total water content

*Since the chemical potential of H_2O in saturated vapour is the same as that in liquid H_2O, thermodynamics predicts this difference should not exist, but it is real nevertheless.

can be classified into "bound" and "free" water [12] and the relative importance of these for permeation and other properties will be found in later sections.

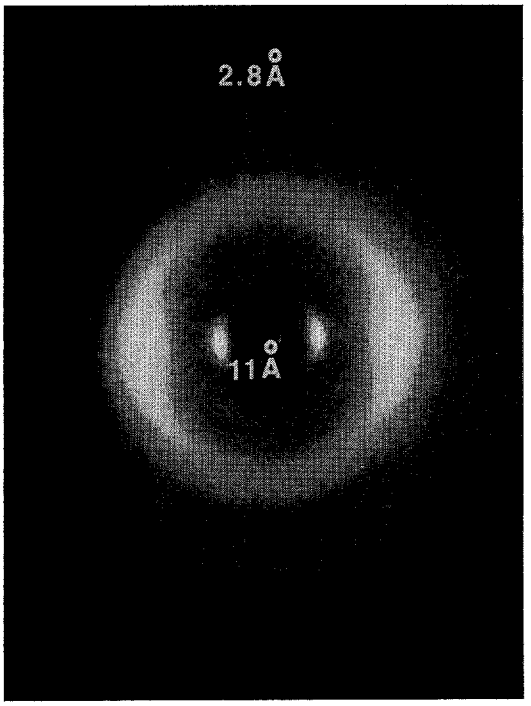

Figure 2 Wide-angle X-ray scattering (WAXS) from dry hollow fibres.

3.3. Wide-angle X-ray scattering (WAXS)

The WAXS pattern from dry hollow fibres is shown in Fig. 2. The excellent orientation of the tropocollagen molecules along the axis of the hollow fibre is evident. This pattern is in no way different from the WAXS pattern of collagen from native tendon [1]. This shows that the main body of the collagen molecule is not affected by enzyme solubilization and restructuring. The two most prominent reflections in the WAXS pattern are the 2.8 Å meridional arc and the 11 Å equatorial spot. The 2.8 Å arc has its origin in the basic repeat distance along the axis of the triple helix of the TC molecule while the 11 Å arc represents the lateral separation of the TC molecules (separation of photofibrils). This reflection is hydration sensitive and has been extensively studied in native tendon by Rougvie and Bear [9]. A similar investigation was undertaken for the hollow fibres and the

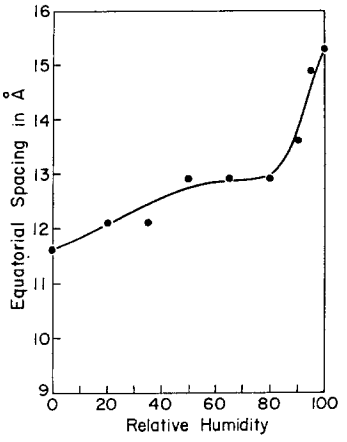

Figure 3 Changes in the WAXS equatorial spacing of the hollow fibres as a function of relative humidity.

equatorial spacings as a function of relative humidity are plotted in Fig. 3. The spacing varies from about 11.6 to 15.25 Å and the shape of the curve is quite similar to the absorption isotherm presented in Fig. 1. This indicates that the spacing increase is due to absorbed water.

Various features of the WAXS pattern are qualitatively similar up to and including 100% R.H. However, if the fibres are soaked in liquid water the WAXS pattern changes drastically. The WAXS pattern from a wet hollow fibre is shown in Fig. 4. Without careful analysis one might even think it to be of an amorphous character. This, however, is not the case. The 2.8 Å arc which represents the fundamental repeat along the axis of the triple helix is still present indicating that the triple helix or at least a portion of it is intact. The excellent orientation of the tropocollagen molecules that was evident in the dry WAXS pattern is decreased. The strong equatorial spots are now very broadened in the equatorial direction indicating that a decrease in fibril size has taken place. In marked contrast to this behaviour, the equatorial spots and the overall features of the WAXS pattern in native tendon become sharper on soaking in H_2O [10].

Assuming that the equatorial spacing changes are due only to absorbed water, one can calculate the volume fraction of water (v_{H_2O}) in the hollow fibres and compare it with values from the absorption isotherms. Such a comparison is shown in Fig. 5. The v_{H_2O} (X-ray) values were obtained by assuming a regular lattice (the exact

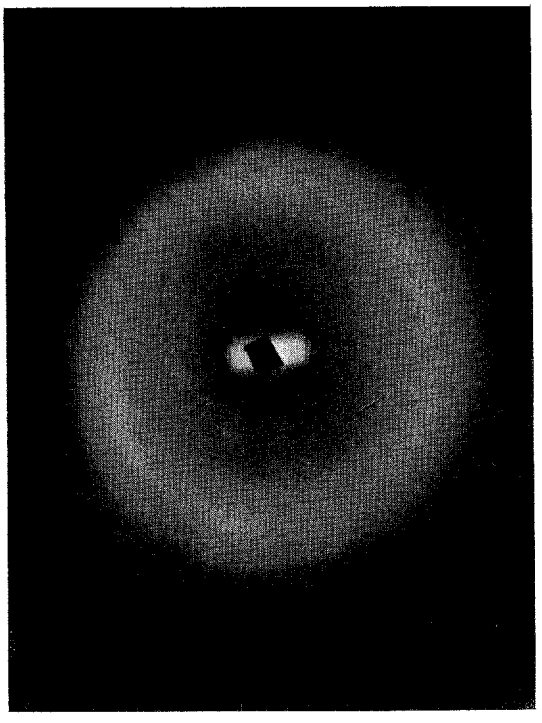

Figure 4 WAXS pattern of wet hollow fibres.

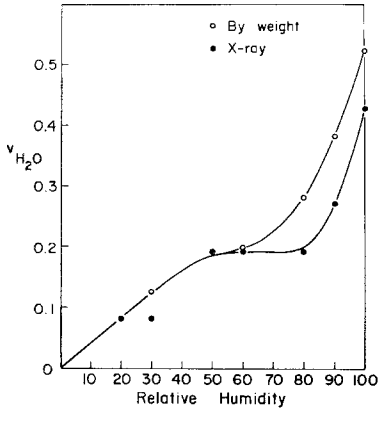

Figure 5 v_{H_2O} (X-ray) and v_{H_2O} (absorption) as a function of relative humidity.

nature of the lattice is not important) with a repeat equal to the equatorial spacing which then is expanded to a new value by the entry of absorbed water. A dry collagen density of 1.35 was used [13]. An examination of Fig. 5 reveals that up to a relative humidity of 60% the two values (absorption and X-ray) for v_{H_2O} are almost identical. Beyond a relative humidity of 60%, the volume fraction measured by absorption exceeds that determined by X-ray measurements. To facilitate easier visualization, the results are replotted in Fig. 6 as v_{H_2O} (X-ray) versus v_{H_2O} (absorption). The absorption measurements measure total water content while X-ray spacing changes measure only that water that is effective in increasing the separation of the protofibrils. We will denote this water as "bound water" and the difference between the two values as "free water". As can be seen from Fig. 6 the amount of "free water" increases as the relative humidity is increased with a levelling off around 100% R.H.

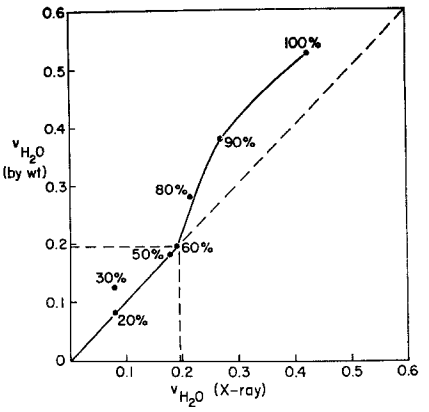

Figure 6 A plot of v_{H_2O} (X-ray) versus v_{H_2O} (absorption) at different relative humidities.

It has already been determined that the total volume fraction water in wet hollow fibres is about 0.85. This gives a value of 0.15 for volume fraction collagen. Using this value and the v_{H_2O} (X-ray) values one can calculate that the v_{H_2O} (bound) is about 0.10, while v_{H_2O} (free) is 0.75 (assuming that no further change in "bound water" occurs on going from 100% R.H. vapour to contact with the liquid).

3.4. Small-angle X-ray scattering (SAXS)

SAXS patterns from dry and wet hollow fibres taken with nickel filtered Cu$K\alpha$ radiation using pinhole collimation are shown in Fig. 7a and b. The equatorial spots in the dry SAXS pattern represent a Bragg spacing of about 48 Å which is approximately four times the WAXS equatorial spacing. This indicates that the periodicity in the lateral direction extends at least a few hundred Å. This reflection is notably absent in the wet SAXS pattern indicating a loss of the long-range lateral periodicity of the protofibrils. The

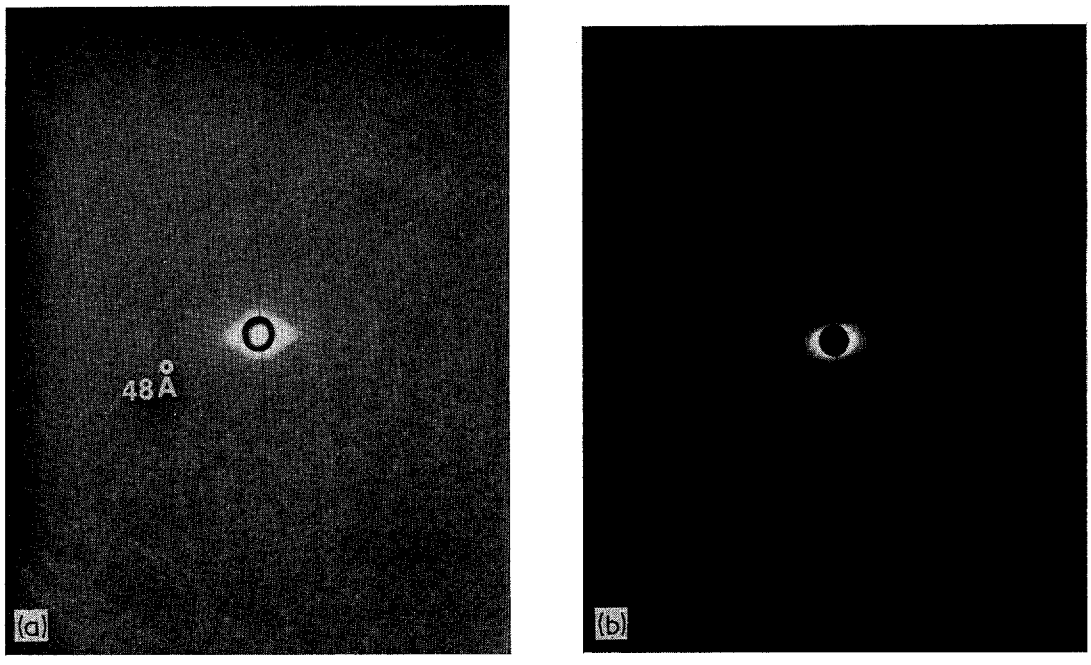

Figure 7 (a) Small-angle X-ray scattering (SAXS) pattern of dry hollow fibres. (b) SAXS from wet hollow fibres.

fibril (a bundle of protofibrils) size may also be decreased.

Also absent in either the dry or the wet SAXS patterns are the meridonial reflections due to the 640 Å periodicity along the fibril axis found in native tendon [14]. These reflections arise primarily from periodic holes in the structure resulting from the quarter staggered arrangement of the collagen molecules [15]. Various structural models as to how such an arrangement can lead to "hole" and "overlap" regions in the fibril have been proposed [16, 17]. Evidently in the reconstituted hollow fibres such holes are absent. However, the 48 Å lateral periodicity of the hollow fibre suggests that a quarter stagger may still be present but without "hole" and "overlap" regions.* The absence of holes could be caused by the removal of the end regions (telopeptides) by enzymatic digestion which might result in a molecule whose length is an integral multiple of the 640 Å periodicity, eliminating the need for "hole" and "overlap" regions.

The strong diffuse intensity around the origin is of considerable interest. It arises chiefly due to long-range electron density differences. SAXS studies on most textile fibres yield similar patterns. In each case the diffuse SAXS intensity has been successfully interpreted as due to microvoids in the structure [18, 19]. In the dry fibres we hypothesize that the scattered diffuse intensity is due to density differences between the fibrils and a small number of voids or pores between the fibrils, while in the wet fibres we believe that the diffuse SAXS is due to differences in electron density between the fibrils and the "free water" present in a much larger number of pores. The wet SAXS pattern differs from the dry SAXS pattern in two respects; it has a smaller aspect ratio of the diffuse intensity, indicating more circular pores or less oriented pore structure and it has a more extensive tail of intensity to higher angles.

If the wet hollow fibre can be considered as a two-phase mixture of fibrils and water, then the SAXS intensity can be analysed to yield quantitative information about the sizes of the fibrils and the "pores" (a pore in this context is a water filled space between fibrils). The analytical tool is Porod's Law [20, 21] which has been extensively

*Strictly speaking a regular array of tropocollagen molecules without holes should give rise to a weaker set of meridonial reflections. In practice these reflections are probably washed out by the strong diffuse scattering extending from the origin. We have examined membranes made partly with native collagen fibres (for strength) and in those membranes we can see evidence of the meridonial SAXS reflections superimposed on the diffuse background.

used to characterize catalysts. If $I(s)$ is the intensity at a scattering vector $\mathbf{s}$ ($|\mathbf{s}| = 2 \sin\theta/\lambda$ where λ is the wavelength of the X-rays and θ is the scattering angle) then Porod's Law dictates that $\lim_{s\to\infty} I(s)\, s^4$ should approach a constant value. This limiting value is proportional to the specific surface area. Another invariant is $\int_0^\infty I(s)\, s^2\, \mathrm{d}s$ which is proportional to the volume of the matter being irradiated. Using these two values one can define a parameter called the "inhomogeneity length" or "correlation distance" l_p, which can be related to the above invariants by the following relations:

$$l_p = \frac{2}{\pi^2} \frac{\int_0^\infty I(s)\, s^2\, \mathrm{d}s}{\lim_{s\to\infty} I(s)\, s^4} \tag{3}$$

and

$$l_p = v_1 \bar{l}_2 = v_2 \bar{l}_1 = \frac{4 v_1 v_2 V}{S} \tag{4}$$

where v_1 and v_2 are the volume fractions of the two phases, V is the total irradiated volume, and S is the total interfacial area. $\bar{l}_1$ and $\bar{l}_2$ are the average lengths of segments of straight lines drawn at random in phase 1 and phase 2, respectively, intercepted by the interface between them.

The parameters $\bar{l}_1$ and $\bar{l}_2$ have been called "range of inhomogeneity" by Porod [22, 23]. It can be seen from Equation 3 that one needs only the relative intensities to evaluate l_p which is a great advantage. The intensities were obtained by photometry using a Joyce Loebl microdensitometer. However, in evaluating the integral $\int_0^\infty I(s)\, s^2\, \mathrm{d}s$ one has available only the intensity values beyond a certain s value defined by the beam stop. The intensity values below this limiting s value were obtained by extrapolating the ln $I(s)$ versus s^2 curves. Although there is an error associated with such a procedure, the contribution of the extrapolated area to the integral is small and the error is not more than 5%. However, the contribution to the above integral from the tail of the scattering curve (large s values) is quite considerable. If it can be established that $I(s)s^4$ does reach an asymptotic value then by making the assumption that $I(s)s^4$ is constant beyond a certain value of $s = s_0$ to $s = \infty$ we can write the complete integral as:

$$\int_0^\infty I(s) s^2 \mathrm{d}s = \int_0^{s_0} I(s) s^2 \mathrm{d}s + \frac{\lim_{s\to\infty} [I(s)s^4]}{s_0}.$$

The wet hollow fibre will be treated as a two-phase system. One phase is collagen with tightly bound water while the other phase is "free water". We have already available to us volume fraction figures of both phases. If l_p can be determined using Equation 3, then $\bar{l}_1$ and $\bar{l}_2$ can be calculated. However, Porod's law is strictly valid only for two phase systems with random orientation. To simulate this condition several hollow fibres (30 to 40) were mounted on a metal frame at different angles and the resulting SAXS pattern was analysed.

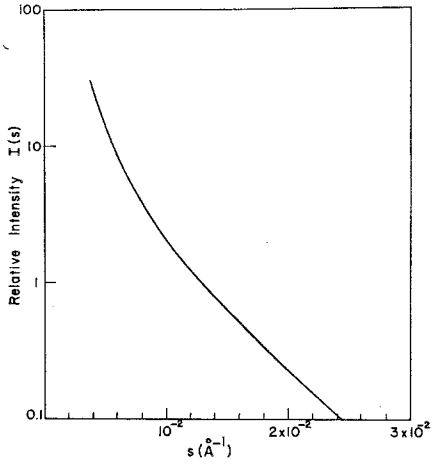

Figure 8 SAXS intensity from wet hollow fibres as a function of scattering angle.

The intensity as a function of scattering angle is shown in Fig. 8. If Porod's Law is to be employed in the analysis of the SAXS data, $I(s)s^4$ must reach an asymptotic value. Fig. 9 shows that this is the case for the wet samples. The limiting values of $I(s)s^4$ were, however, quite different for dry and wet hollow fibres, the limit being much larger for the wet than the dry fibres. This indicates that a large amount of new surface has been created by entry of water into the HF. The l_p values obtained were 160 Å for the dry hollow fibres and 40 Å for the wet hollow fibres. Estimating $\bar{l}_1$ and $\bar{l}_2$ for the dry hollow fibres requires a knowledge of the volume fraction of pores. Although this is not available to us experimentally, we assume a value for $v_{\text{pores}} \approx 0.10$. Using this value and Equation 4

$$\bar{l}_{\text{collagen fibrils}} \approx 1600 \text{ Å}$$

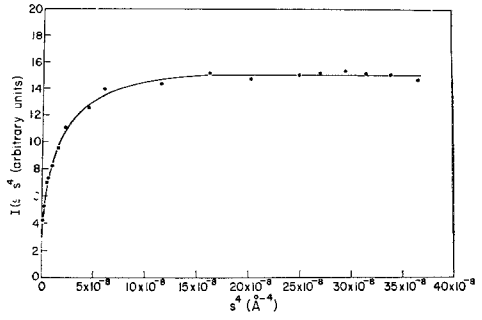

Figure 9 Porod's Law behaviour of the tails of the SAXS curve from the wet hollow fibres.

and

$$\bar{l}_{\text{pore}} \approx 170\,\text{Å}\,.$$

Fig. 10 shows a scanning electron micrograph of a dry hollow fibre fractured in liquid nitrogen (courtesy Dr Teruo Miyata). It can be seen that the fibrils have excellent orientation along the axis of the hollow fibre and the size of the fibrils is about 1500 Å in good agreement with the above value. Thus the assumption of 10% pores seems to be reasonable.

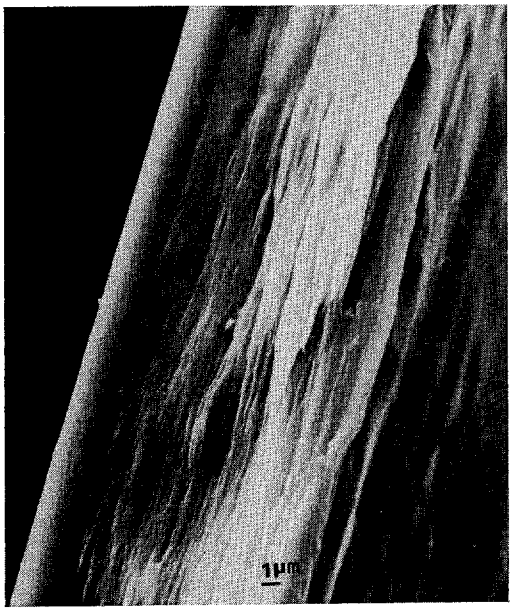

Figure 10 Scanning electron micrograph of dry hollow fibres fractured in liquid nitrogen (courtesy of Dr T. Miyata).

In the wet hollow fibre v_{H_2O} (free) is 0.75. Using this value we find

$$\bar{l}_{\text{fibrils}} \approx 60\,\text{Å}$$
$$\bar{l}_{\text{water}} \approx 160\,\text{Å}\,.$$

The aspect ratio of the wet SAXS intensity indicates that the pores are elongated along the axis of the fibre. The above $\bar{l}$ values for the fibrils and pores represent approximately the smallest dimension of the fibril or pore (transverse to the axis of the hollow fibre).

4. Discussion and conclusions

The SAXS results indicate that the fibril size is greatly reduced (from ~ 1600 to 60 Å) by saturation with water. This decrease in size of the scattering units may be partly responsible for the absence of the 48 Å equatorial reflection and the smearing out of the ~ 12 Å equatorial reflection in the WAXS pattern of the wet hollow fibres. The H_2O molecules which enter the cross-linked collagen structure logically will be concentrated in regions between the cross-links and the cross-links will define the length of the pores so created. This absorption of H_2O into pores between cross-links must necessarily result in some disorientation of the fibrils. The consequences of such disorientation can be observed macroscopically. Upon wetting, the lateral dimensions of the hollow fibre (thickness and internal diameter) increase considerably while the hollow fibre shrinks slightly along its length, the direction of original fibril orientation. Using all this information a model of the structural changes that take place upon wetting in the hollow fibre can be presented and is shown schematically in Fig. 11.

The disorientation of the fibrils introduced by water can be removed to some extent (and the aspect ratio of the pores increased) by stretching the hollow fibre. To prove this point SAXS patterns were taken on single hollow fibres with and without tension. The results are shown in Fig. 12a and b. The SAXS pattern under tension shows an increased pore aspect ratio in the reciprocal aspect ratio of the diffuse scattering.*

Measurements of mechanical and transport properties of the wet hollow fibre give further credence to the above model. These results will be published in a subsequent paper. Besides aiding in the interpretation of the physical

*It is difficult to quantitatively interpret these changes because of the difficulties discussed above in analysing the diffuse scattering from oriented systems as well as our lack of knowledge of v_{H_2O} (free) under tension.

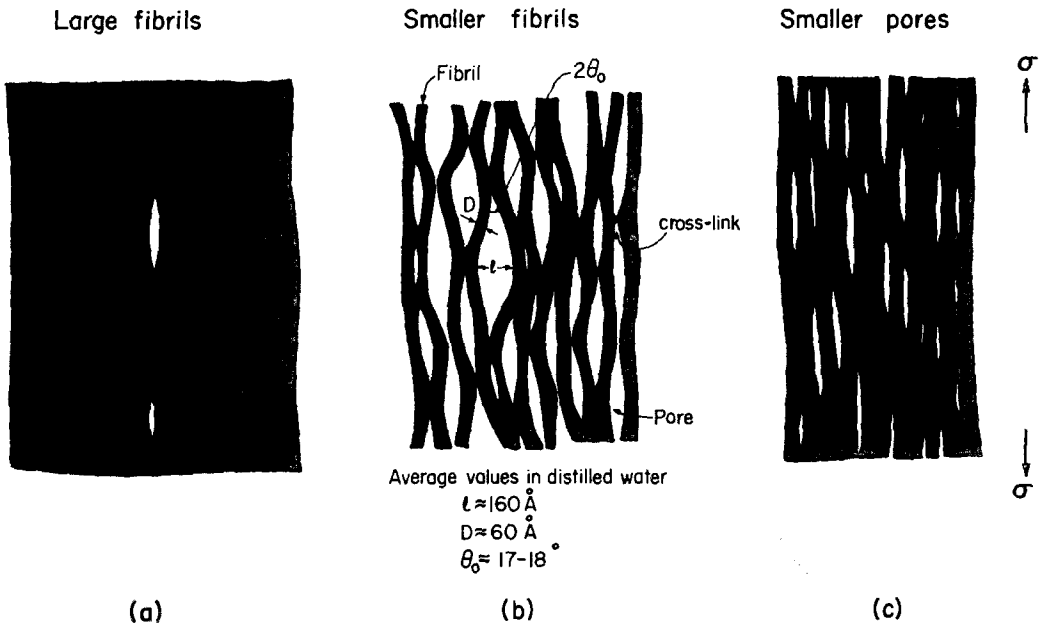

Figure 11 Schematic diagram of sections through the wall of a hollow fibre indicating changes in the structure of the fibres upon entry of water. (a) Dry, (b) wet, (c) wet with tension.

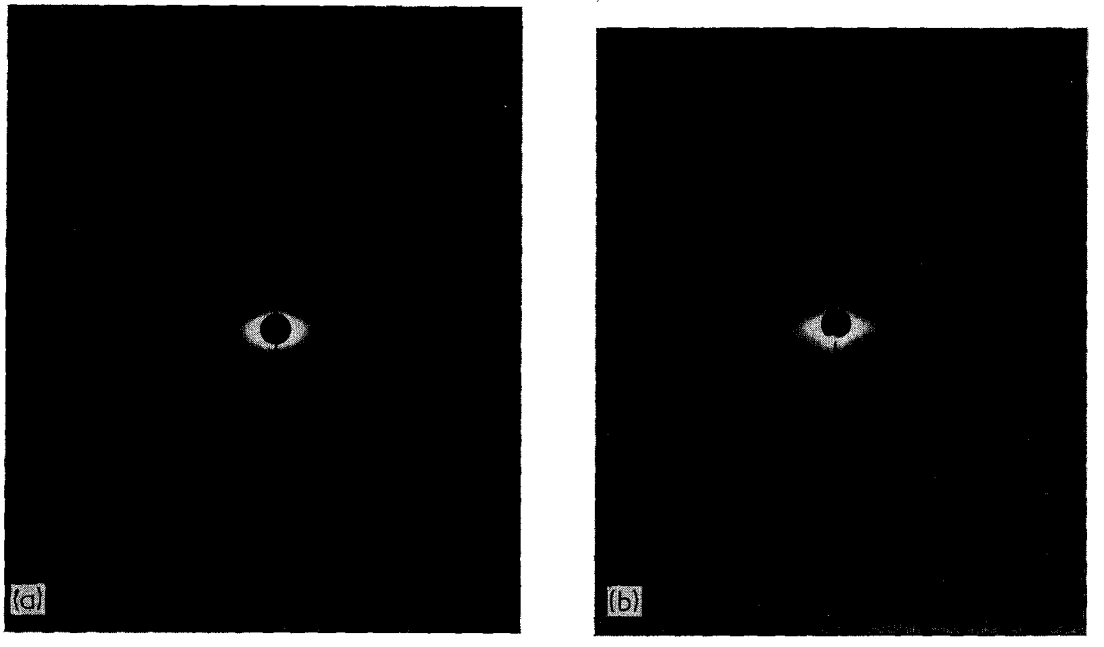

Figure 12 (a) SAXS from a wet hollow fibre without tension. (b) SAXS from a wet hollow fibre with tension.

properties that relate to the pore structure, the model may be useful in predicting the effects of processing variables (such as ultra-violet irradiation time) on the pore structure. We expect that further SAXS studies, interpreted on the basis of this model, will lead to a better understanding of the effects of these processing variables on the structure and properties of collagen membranes.

Acknowledgements

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