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Small angle X-ray fibre diffraction on aligned elongated macromolecules has been a routine tool for the structure of materials that either can not be crystallized or in cases in which one wants to study the structure of the molecule as close as possible to those occurring in solution. One of the problems with this approach is that quite often it is not possible to obtain completely aligned samples but only samples in which there is an angular distribution around the main orientation axis. This has as a consequence that at larger scattering angles the contributions from different layer lines start to overlap. A possible method to overcome this problem can be found in combining results from small angle solution scattering with small angle fibre diffraction from molecules aligned with their long axis parallel with respect to the X-ray beam. In the latter case one only observes the projection of the molecule on the basal plane. We show how this method can be applied to hydrated microtubules.

1. Introduction

Although many structural aspects from biopolymers have been elucidated by fibre diffraction, protein crystallography or electron microscopy it is still difficult to obtain high resolution structural information from them in solution, which is the normal environment for biologically active materials. Although SAXS and SANS have been used in many cases to study these polymers the loss of information due to the orientational averaging poses in some cases problems. Fibre diffraction on oriented molecules in solution can add information normally not accessible by solution scattering.

Besides the occurrence of spontaneous orientation [Flory, 1956] the most common methods to prepare oriented samples are shearing and the application of external magnetic or electric fields. Static electric fields in general are not very successful and one has to use pulsed fields which creates an extra complication [Koch et al., 1995]. Shearing is most times more effective but even that is not always a successful method especially with long rather fragile molecules [Buxbaum et al., 1987]. In some cases one can utilize strong magnetic fields in which the interaction of diamagnetic polymer units with an applied magnetic field forces the molecules to orient [Torbet, 1986; Bras et al., 1998]. However, the diamagnetism of biopolymers is often quite weak and one has to use very strong fields. In some cases combinations of different alignment techniques, like for instance magnetic fields in combination with slow speed centrifugation can produce good results [Yamashita et al. 1998].

In general these methods do not provide samples with a perfect orientation but the molecule axes will exhibit a spread. The result of this is that in the diffraction pattern the intensities are spread out into an arc. In the analysis of data this can provide problems when, at higher scattering angle, the diffraction arcs of different layer lines start to overlap so that, to assign the real diffraction intensities to the peaks, one has to deconvolute the different contributions. Using the correct algorithms and assumptions this is sometimes possible but it is a method that is fraught with uncertainty as to the correctness of the results especially if the degree of orientation is fairly low.

Microtubules are long hollow cylindrical molecules which have a multitude of functions in the cell, especially during cell division which makes them a primary target for anti-cancer drugs [Robert and Hyams, 1979]. They consist of long so called protofilaments in which tubulin dimers are connected in a head to tail fashion. These protofilaments connect laterally, but slightly staggered with respect to each other, to form the hollow microtubule. The diameter of the cylinder depends on the number of protofilaments but in the most common case, in which 13 protofilaments make up the cylinder wall, the outer diameter is approximately 150 Å and the inner 90 Å. Recent structural studies using reconstructions on the basis of electron microscopy have elucidated the secondary structure [Nogales et al, 1995]. In the assembled microtubule the protofilaments are slightly staggered with respect to each other which results in a surface that is modulated by several helical grooves. The length of the microtubules can extend up to several μm while the persistence length has been reported to be above 2 μm [Venier et al., 1994; Gittes et al., 1993], which makes this molecule an archetypal rigid rod system.

Microtubules in solution are in a dynamic equilibrium which means that they continually assemble and disassemble. Shearing has indeed shown to produce some alignment but only in small domains in a further isotropic environment [Buxbaum et al., 1987]. Another method of alignment is to centrifuge them at high speeds for extended periods but this has the unwanted side effect that highly concentrated dehydrated samples are created [Mandelkow, 1986]. Using the method of assembly in high magnetic fields we have been able to produce hydrated samples in which the molecules are uniformly aligned and in which the concentrations are still so low that inter-microtubule interactions are not imposed by a

small free volume but are similar to those encountered in the natural environment [Bras et al., 1998].

However the drawback of this method is that the alignment is not very high which makes high resolution structural studies unfeasible. By combining information from scattering patterns from microtubules oriented with their axes normal to the X-ray beam with similar patterns from polymers aligned parallel to the X-ray beam one can extract information from the diffraction pattern which is normally not possible. The fibre diffraction pattern consists of discrete layer lines on which the distance between the meridian and the first diffraction intensity on a layer line gradually increases with layer line number; the well known X-pattern associated with helical symmetry. This, in combination with the application of helical diffraction theory [Amos and Klug, 1974] and solution scattering data, allows us to solve some problems created by the low degree of alignment and overlap of the contributions from different layer lines. This method can be applied to other systems as well.

By using samples which are oriented with their long axis parallel to the X-ray beam it is also possible to study more accurately the effects of the lateral interactions between the aligned molecules whose concentration dependent effects are observed at very low-q values. In a slightly disoriented system one has to make a judicious judgement over which arc to integrate the data in order to determine the accurate intensities corresponding to the different diffraction features. This problem is circumvented in this way since we are now dealing with a circularly symmetric pattern which allows a much more accurate determination of the low-q scattering intensity.

2. Materials and Methods

The protein tubulin was purified from pig brains and prepared for experiments as described elsewhere [Andreu et al., 1992]. The protein concentration was determined spectrophotometrically. For assembly the tubulin was equilibrated with 10 mM sodium phosphate, 3.4 M glycerol, 1 mM EDTA, 0.1 mM GTP buffer (pH 7.0) after which 1 mM GTP and the desired concentration of $MgCl_2$ were added. The samples were centrifuged for 10 minutes at 50,000 rpm at 4° C to eliminate aggregates. Assembly could be initiated by raising the temperature from 4° C to 37° C. With the biochemical methods used in this work the average length and molecular weight are 5 μm and 10^9 Dalton, respectively.

Small Angle Diffraction and Scattering patterns were obtained on station 2.1 from the Synchrotron Radiation Source Daresbury. This station is described elsewhere extensively [Towns-Andrews et al., 1989]. The main details are that the radiation used had a wavelength of 1.5 Å and the flux was approximately 10^{11} photons/sec. The samples were placed in cells with mica windows in order to reduce background scattering. To avoid radiation damage elongated cells were used which were slowly translated through the X-ray beam.

The samples were aligned by assembling them in a 9 Tesla magnetic field generated by an Oxford Instruments superconducting magnet. Further details of the magnetic alignment method used for these polymers are discussed in [Bras, 1995; Bras et al., 1998].

3. Results

Using the magnetic alignment method fibre diffraction patterns have been obtained which show a partial orientation in which the spread of the microtubule axis with respect to the main axis is approximately 11 degrees. See figure 1.

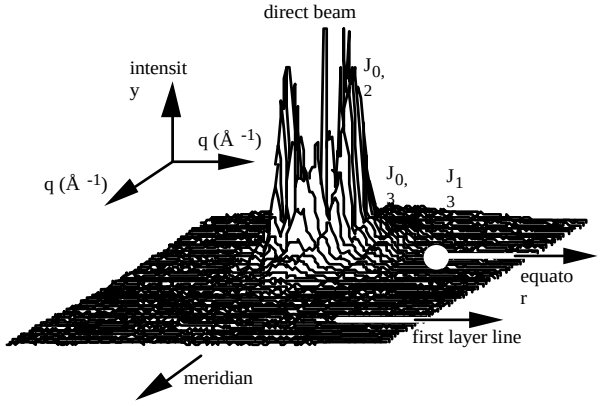
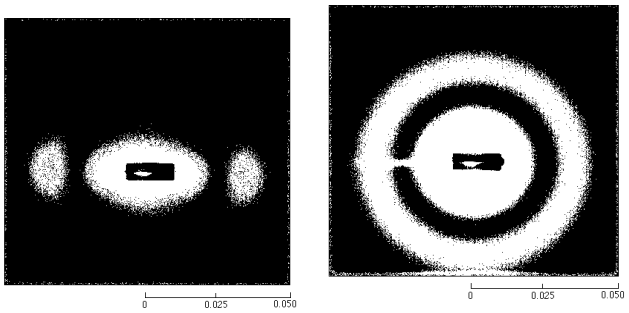


Figure 1
Three dimensional representation of a small angle fibre diffraction pattern of microtubules aligned by assembling them inside a 9 Tesla magnetic field. Once aligned the long microtubules are inhibited to disorient due to steric hindering. The pattern here is an averaging of approximately 4 hours of data collection on different samples. This is necessary due to the low electron density difference between the microtubules and the buffer solution. The ratio of the highest to lowest diffraction peak in this pattern is 10^4:1 with the weaker peaks situated on the layer lines.

Examples of small angle fibre diffraction patterns from samples aligned with their axis parallel and at right angles with respect to the X-ray beam are shown in figure 2.

When the samples are in the parallel configuration one observes the molecular electron density contrast projected on the basal plane of the molecule. This is equivalent to an unoriented solution scattering pattern in the scattering vector ($q=2\pi/d$) range in which no overlap between the layer lines and the equatorial diffraction trace can be expected. At higher q-values, where, in a solution scattering experiment, the layer lines contributions will start to overlap with the equatorial the diffraction pattern from the parallel oriented samples still should only exhibit the features corresponding to the equatorial intensities up to the region where the higher order layer lines intrinsically start to show overlap due to the curvature of the Ewald sphere.



A **B**

Figure 2
Low resolution scattering patterns of microtubules aligned with their axis parallel (panel A) and perpendicular (panel B) to the X-ray beam.. (Scale is in $Å^{-1}$).

The scattering pattern obtained in the parallel configuration can be expressed in terms of Bessel functions describing a hollow cylinder. These functions are modulated by the scattering pattern due to the elongated indentations, parallel to the long molecule axis, between the protofilaments both on the inner and outer wall of the hollow microtubule. These grooves will give rise to diffraction intensity described by a $J_n(qR)$ Bessel function in which n reflects the n-fold symmetry of these grooves. In this case n is assumed to be an integer multiple of 13, and R is the average radius around which the grooves are undulating. From helical diffraction theory we can find that the J_{13} Bessel function in this case will not show any intensity below q= 0.095 $Å^{-1}$ so that the first three peaks can be ascribed to the function describing the hollow cylinder [Vainshtein, 1966].

$$F(q) = R_{outer}\frac{J_1(qR_{outer})}{q} - R_{inner}\frac{J_1(qR_{inner})}{q} \qquad (1)$$

Excluding the central region where interparticle scatter also contributes to the scattering pattern, these peaks, up to the first J_{13} maximum, can be used to accurately determine the inner and outer radius of the polymer by fitting the experimental data to equation (1). These are determined to be 86 ± 5 Å and 146 ± 5 Å, respectively. Keeping these parameters fixed and extending the fitting range to include the first J_{13} maxima from the outer cylinder wall and adding this function to the fitting function we can determine the radial extent of the electron density grooves. See figure 3. By this method we find that the grooves on the surface are 21 ± 4 Å deep. This procedure can then be iterated to include the J_{13} maxima from the inner cylinder wall. Unfortunately accurate experimental data in this range are not available yet. However, the procedure to be followed next in order to accurately determine the scattering intensity on the first layer line is to compare the scattering trace determined in this way with unoriented solution scattering data obtained in the range where both the equatorial scattering intensity and the first layer line intensity contribute to the solution scattering pattern. See figure 4. This procedure can be iterated to include the next layer line. A maximum on the resolution that can be achieved in this way is due to the curvature of the Ewald sphere so that layer lines intrinsically start to overlap with the equatorial intensity. This is around $q \approx 0.16$ $Å^{-1}$.

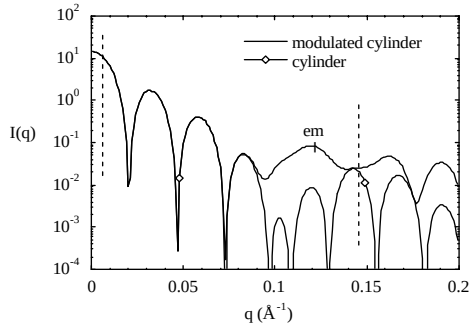


Figure 3
The fitted curves describing the equatorial diffraction intensity. The extent of the experimental data and the fitting range is indicated by the two dotted lines. The bar indicated by 'em' is the maximum deviation with respect to the experimental data. This deviation is comparable with the experimental error in the data.

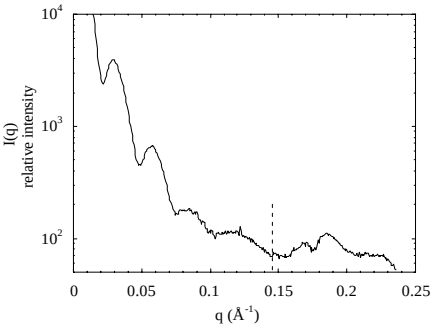


Figure 4
Solution scattering pattern from randomly oriented microtubules. The intrinsic background has not been subtracted. Beyond the dotted line the overlap from the first layer line with the equatorial diffraction intensity occurs. From comparisons with figure 3 it will be clear that the contribution of the layer line to the total intensity is small and that thus an accurate determination of this intensity is difficult. Solution scattering data was made available by J.M. Andreu.

Another aspect of these experiments is that the interparticle interactions on a statistical large ensemble of polymers can be studied. Figure 5 shows the concentration dependent experimental scattering curves at small scattering vectors from samples aligned with their axis parallel to the X-ray beam, where the interparticle scattering structure factor adds scattering intensity to the form factor of the microtubules.

The interference effects were modeled by considering the sample as a two-dimensional disordered fluid. The microtubules were represented as hollow discs with an inner radius of 86 Å outer radius of 146 Å. These discs were placed randomly in a square with sides 10 000 Å. The average distance between the centres of the discs was varied by changing the number of discs. The packing of N discs into the box was carried out under the constraint that the distance r_{nm} between the centres of any two discs n and m avoided unphysical overlap. The initial configuration of the discs in the box was changed by allowing each disc to undergo a random displacement with a maximum amplitude of 16 Å. The disc to be moved was chosen at random. A new configuration was produced after attempting a displacement

of every disc in the box. The simulation proceeded by generating up to 16 000 configurations and evaluating the diffracted intensity I_L using the positions of the disc centres for every fifth configuration. I_L was calculated using the expression $I_L(\bar{q}) = I_D(\bar{q}) \sum_{n,m} exp[-i\bar{q}.\bar{r}]$ where I_D is the scattered intensity from single hollow discs and q is the wave vector. In practice, only $I_L(q_X)$ was calculated, as the scattered intensity was found to be symmetric in the $\{q_x, q_y\}$ plane as expected. We evaluated the scattered intensities from configurations containing between 50 and 700 discs, covering an average nearest centre-to-centre distances between 1000 Å and 330 Å. The number of configurations used was chosen judiciously so as to yield statistical fluctuations of less than 3% in the diffracted intensity.

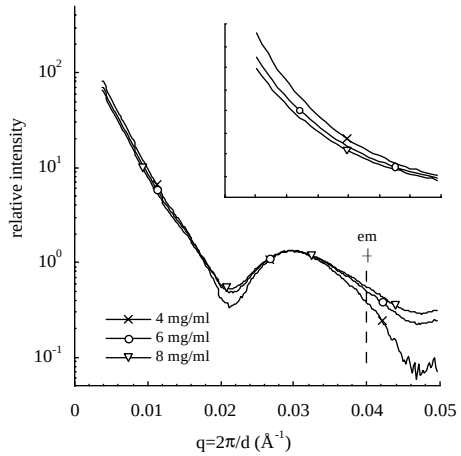


Figure 5
Low resolution scattering data from microtubules oriented parallel with the X-ray beam. The inset shows the very low q-range on a linear scale. The effects of interparticle scatter are visible.

When the concentration dependent interparticle effects were simulated it was found that these effects are much stronger that can be expected from samples with a random distribution in the plane. This points towards pattern formation in which the polymers are on average arranging themselves in bundles, thus creating locally higher concentrations. To model this behavior it was assumed that there exists an attractive potential between the aligned microtubules. Two types of potentials, a Lennard-Jones and a square well potential, were tested out. See figure 6. The Lennard-Jones potential proved to be less suitable since the calculated structure factor interferes strongly with the form factor scattering and thus creates a scattering pattern not compatible with the experimental data. The square well potential was more successful explaining the data. This is due to the fact that the first intensity maximum of the structure factor is located at the position of the first minimum of the form factor scattering, thus reducing it's effects considerably. At higher q-values the structure factor gently undulates and only slightly distorts the form factor so that when comparing this to the experimental data it falls within the error margins. In nature there are many applications in which microtubules form bundles. This is generally caused by the interactions of microtubule associated proteins (MAP's) which stick out of the polymer wall and interact with neighboring polymers. However, we have used a preparation in which these MAP's were removed so that we can state that in this way it is possible to study the pure interaction between aligned microtubules. This interaction can be modeled by a short range square well potential.

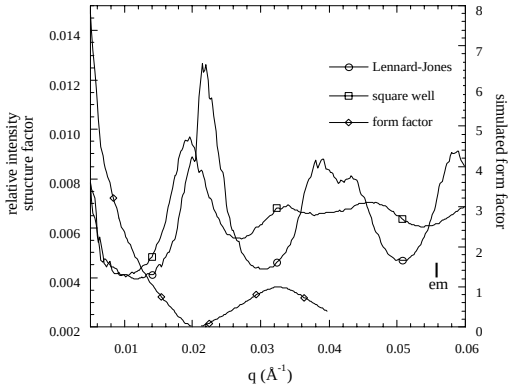


Figure 6
Form factor and structure factor for simulated data sets. The short range square well potential represents the experimental data more accurately. The long range Lennard-Jones potential distorts the form factor scattering too much.

By using a method which produced very short microtubules we managed to obtain higher degrees of alignment (≈ 6°) but once exposed to the X-ray beam these samples broke up in different domains of approximately 1 mm with alternating orientations. The different domains had an orientation of ≈ 45° with respect to each other [Bras, 1995]. Interestingly this domain formation occurred throughout the cell, even in the regions not exposed to X-rays. There is a slight resemblance with the spontaneous pattern formations from microtubules in oscillating assembly/disassembly conditions, but in that case the authors reported orientations of the different domains of 90° [Tabony and Job, 1990]. No further investigations on this interesting phenomenon have been performed yet. Since some aspects of the biological functions require that these molecules form bundles it is clear that more detailed knowledge of the inter-microtubule interactions deserves would be most welcome.

Conclusions

We have shown that from a judicious combination of fibre diffraction and solution scattering accurate diffraction intensities can be determined even for polymers whose degree of orientation is not very high. Also the interparticle interaction between oriented molecules can be studied. Obviously these results don't have to be limited to microtubules alone but can be applied to any polymeric solution from which oriented samples can be prepared.

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