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Dynamic flow alignment of flagellar axonemes for low-angle X-ray fiber diffraction analysis

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Introduction

Polymer and filamentous structure is one of the most common features of biological materials, for example, the strands of DNA/RNA molecules and the chains of α-helix/β-sheet structures of protein molecules. We can also find out many other specific types of biological fibers assembled from unit peptide monomers, e.g., the thin filament of actin, microtubules of tubulin and intermediate filaments of vimentin/cytokeratin as intracellular cytoskeleton, as well as collagen and fibronectin as extra-cellular filaments. Other types of filaments such as silk and spider fibers are of interest as material of high tensile strength. Amyloid fibrils associated with the pathological conditions of Alzheimer's disease or bovine spongiform encephalopathy are of medical significance.

The structure of these biological filaments and associated components is closely related to their functions and properties, however, the investigation of structural details is not easy task due to the difficulty of crystallization of such long fibers. Alternatively, high-energy synchrotron X-ray fiber diffraction is a powerful approach to know fine structures at sub-nanometer scale under physiological conditions. For the X-ray fiber diffraction, however, we need to solve a crucial problem, i.e., how to make filaments assembled and aligned in the same orientation. The diffraction signals corresponds to the rotational averaging of diffractions coming from longitudinal regularity along filaments, but the angular misalignment of fibers obscures diffraction signals and structural details cannot be obtained.

To improve the quality of X-ray fiber diffraction data, several techniques have been used so far, e.g. slow sedimentation with low g centrifugation or/and biased Brownian motions under quite a strong magnetic field as high as several Teslas [1-4]. These methods required a long time for the preparation of specimen from several hours to a few weeks to accomplish fiber alignment and the results are not always reproducible. In addition, we cannot use the same technique for many different types of biological filaments since they become denatured quickly at room temperature or some physiologically active components are easily degraded. In the present study, we modified an instrument for rheometry and developed a quick and reproducible technique for fiber alignment. We applied the technique for the X-ray diffraction analysis of isolated flagellar axonemes of sea-urchin spermatozoa.

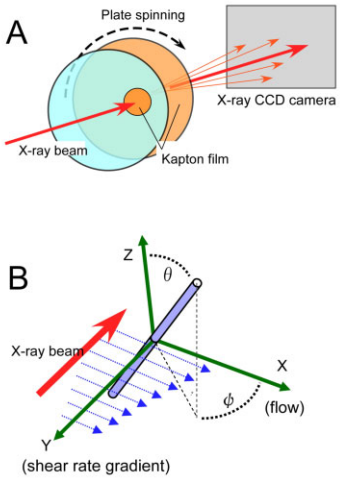

Fig.1 Schematic drawing of the apparatus for X-ray diffraction experiment. (A) Specimen was placed between two parallel discs, one of which was rotated with a constant rate (0-5 s^{-1}) Through a chamber window made of Kapton film, X-ray beam was introduced through the specimen suspension and diffraction signals from aligned filaments were recorded with a Hamamatsu cooled CCD camera (camera length, ca. 1m). B illustrates the relationship between the X-ray beam path and flow-rate gradient given to the specimen.

Material and Method

Sperm was collected by injecting 0.55 M KCl into the body cavities of male sea-urchins (Hemicentrotus pulcherrimus). The collected sperm was kept refrigerated and used for experiment in three days. Ten ml of the collected sperm was first diluted with 0.55M NaCl and subsequently cell membrane was extracted for one minute with a 100 ml solution containing 0.04 % Triton X-100, 0.4 M K-acetate, 1 mM ethylene glycol-bis-(beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 10 mM Tris-HCl (pH. 8.3) on ice. After demembranation, spermatozoa were collected by centrifugation (4,000 g) and slow 30-40 pestle strokes in Dounce homogenizer were given to remove sperm heads mechanically. After removing head with slow centrifugation (4,000g), axonemal fragments were collected with 8,000g centrifugation for X-ray diffraction analysis.

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For flow-induced alignment of axonemes, we used an instrument as schematically illustrated in. Fig. 1. Specimen suspension containing the demembranated and fragmented axonemes were placed between two parallel discs. The volume of chamber was ca. 70 μl. Then, one of the discs was rotated with constant rate (<5 s-1) to give a constant shear gradient to the suspension of axonemes.

For the observation of X-ray diffraction pattern, we used monochromatized X-ray beam (0.5 mm (V) - 1.5 mm (H); wavelength of 0.15 nm) selected and collimated from synchrotron radiation emitted from the beam line BL15A port of the positron storage ring at the Photon Factory, KEK (Tsukuba, Japan). The X-ray beam was introduced into the specimen as shown in Fig. 1 through a Kapton film (thickness, 0.05mm). Diffracted X-ray signals before and during disc rotation was recorded with a cooled CCD camera (exposure time, 8 sec). Recorded images were analyzed with ImageJ (ver.1.34s, NIH).

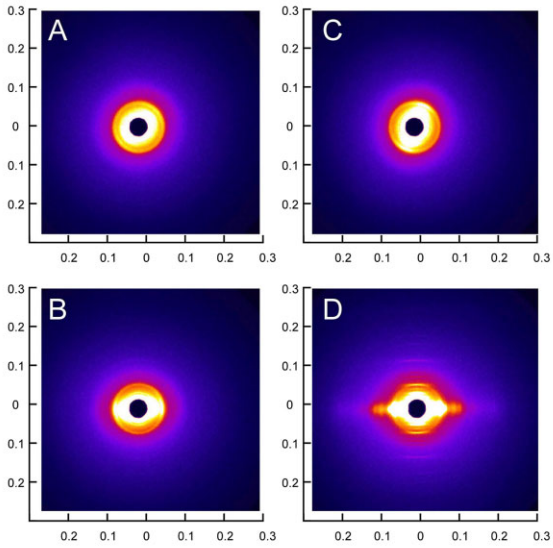

Fig.2 X-ray diffraction from sea-urchin sperm axonemes. Axonemes are placed between two parallel discs shown in Fig.1 and flow shear was given. Horizontal and vertical axes correspond to Z and X axes in Fig. 1. Diffraction signals in the range of spatial frequency 0-0.3 nm^{-1} are shown. A, B without methylcellulose. C, D with methylcellulose. In the case of D, flow-depending alignment of axonemes was clearly observed (angular deviation of about 8 degrees). The regular arrangement of structural units in meridional and equatorial directions, including tubulin molecules (0.25 nm^{-1}) is evident.

Results and Discussion

Axonemes have around 500 peptides that are regularly arranged along the longitudinal axis. Main components that would diffract X-ray are tubulin (4, 8 or 16 nm spacing) and dynein arms (24 or 96 nm spacing) that compose ca. 90% of axonemal protein. These diffraction signals are good indicates to show how axonemal filaments are aligned well to the flow (X-axis, Fig. 1B) or flow vorticity axis (Z-axis) given to the specimen by spinning of the parallel discs (Fig. 1).

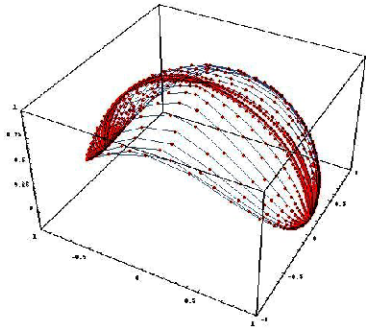

Fig.3 Rods under shear-rate gradient show cyclic motion called Jeffery orbits. An example of calculated orbits (starting from $\theta = \pi/2$ at t=0) is shown. Lines show the trajectory of motion. Red dots indicate the position of rod ends at a constant time interval.

Fig. 2 shows the results. Before we started disc rotation, there were no specific diffraction spots in a specific direction. It indicates axonemal orientation was random in the suspension. Soon after we started the disc rotation, a specific diffraction pattern appeared. As shown in Fig. 1D, clear diffraction signals of 0.125, 0.0625 and 0.041 nm-1 (corresponding to 8, 16 and 24 nm periodicity) were observed as sharp layer lines in the flow (X-axis) direction. Two spots of 0.25 nm-1 axial repeats were clear and it indicates helical arrangement of tubulin molecules in axonemal microtubules. We concluded that axonemes were aligned well in the direction of flow (X-axis), since all these meridional signals are expected from longitudinal regular arrangements of axonemal components. Specific diffractions, probably coming from 9+2 arrangement of microtubules or the microtubules diameters were observed as the equatorial spots (Fig. 2D). Since these equatorial signals are coming from the convolution summation of each equatorial diffraction spot, we could estimate the angular deviation of fiber alignment. It was ca. 8 degrees in the case of Fig. 2D.

We found that including methylcellulose in the medium of axonemal suspension was quite effective to facilitate the flow-induced alignment. Without methylcellulose

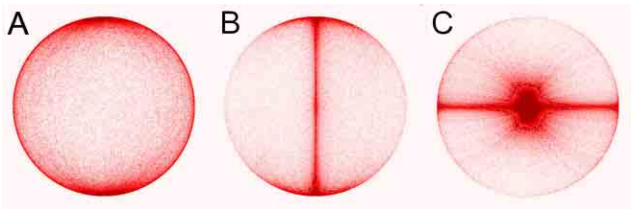

Fig.4 Diagram showing the calculated distribution of filament ends of axonemes aligned under flow-shear gradient. Calculation was carried our as shown in Fig. 3. A, B and C show the results in XZ (horizontal, Z-axis), XY (horizontal, Y-axis) and YZ (horizontal, Z-axis) planes (Fig.1B), respectively. Calculated mean angles and deviations are 0.65±4.8, 0.07±2.9 and 20.7±24.5 degrees.

(Fig. 2B), the angular deviation of aligned axonemes was around 20 degrees. We could not improve the fiber alignment under the3 same condition by the prolonged application of flow-shear. On the other hand, with methylcellulose, flow induced alignment was observed immediately after the start of shearing and in a shear-rate depending manner.

Under the present condition with a flow rate gradient, rods suspended in the medium should show specific cyclic motion called Jeffery orbits [5, 6] as described by the following equations.

$$\frac{d\varphi}{dt} = \frac{\dot{\gamma}}{p^2+1}(p^2\cos^2\varphi + \sin^2\varphi)$$

$$\frac{d\theta}{dt} = \frac{\dot{\gamma}}{4}\left(\frac{p^2-1}{p^2+1}\right)\sin 2\theta \cdot \cos 2\varphi$$

where, θ and ϕ are the angle of suspended rod as shown in Fig. 1B, p is the aspect ratio of filaments (ca. 400) and $\dot{\gamma}$ is the shear rate.

Fig. 3 shows an example of calculated Jeffery orbits. When rods are parallel to XY plane (in the plane of flow and shear-rate-gradient), they show cyclic rotation in XY plane, but mainly stay aligned in the X (flow direction) direction. When rods are in XZ plane (perpendicular to the flow-rate gradient), they stay in XZ plane with almost random orientations. Such behavior of suspended rods in Newtonian fluid is summarized in Fig. 4.

Angular deviation of rods calculated with Jeffery orbits is around 4.8 degrees in XZ plane (Fig.4A), which would be almost comparable to 8 degrees of flow-aligned axonemes in the medium containing methylcellulose (Fig. 2D). One of the possible interpretations is that it would require quite a long time for rods to show the stable and equilibrated distribution of angles and that methylcellulose would accelerate the effects. In the case we did not add methylcellulose (Fig. 2B), shearing with several second seems to be not long enough for such equilibrium.

It has experimentally been shown that rods did not show simple Jeffery orbits in non-Newtonian fluid [6, 7]. Fluid elasticity and viscosity would be working in a complex way in the case of non-Newtonian medium. The effects of methylcellulose we observed in the present study would reflect such complicated shear effects on the rod alignment partially coming from the shear-thinning effects on medium.

The present study provides us with a novel and powerful technique for the field of fiber diffraction as follows. (1) We only need put specimen suspension between two discs and start spinning. Quick surveys of diffraction patterns and structural analysis can be executed. (2) The quality of alignment was better than or almost equivalent to those with conventional techniques [2-4]. (3) A small volume (10-100 μl) of specimen is enough for the analysis. We can apply the technique to many other biological materials even in a case quite a small amount of specimen is available.

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References

[1] F. Family et al., Phys. Rev. Lett. 84, 9999 (2000).
[2] S. Author et al., J. Biol. Chem. 274, 8888 (1999).
[1] G. Stubbs, Curr. Opin. Struct. Biol. 9, 615-619 (1999).
[2] I. Yamashita et al., J. Mol. Biol. 278, 609-615 (1998).
[3] T. Oda et al., Biophys. J. 75, 2672-2681(1998).
[4] W. Bras et al., Biophys. J., 74, 1509 (1998).
[5] C.J.S. Petrie, J. Non-Newtonian Fluid Mech., 87, 369 (1999).
[6] Y. Iso et al., J. Non-Newtonian Fluid Mech., 62, 115 (1996).
[7] Y. Iso et al., J. Non-Newtonian Fluid Mech., 62, 135 (1996).

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