

Use of Microcoil Probehead for Determination of the Structure of Oriented Silk Fibers by Solid-State NMR

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A microcoil probehead for solid-state NMR was developed with a two-channel radio-frequency circuit, and ^{13}C observation with a proton-decoupling probehead was performed to obtain information on the distribution of the orientation of silk fibroin molecules in the fiber. The coil (1 mm (diameter) \times 5 mm (length)) of the probehead was placed at the angles 90° and 30° , relative to the static magnetic field. Only $70\ \mu\text{g}$ of $[1-^{13}\text{C}]$ -Gly silk fibroin fiber was used in a magnet of 9.4 T (400 MHz for proton channel).

Introduction

Solid-state nuclear magnetic resonance (NMR) is one of the most powerful analytical tools in the fields of materials science and life science. The weakest point in the analysis is its low sensitivity. Therefore, large amounts of samples are required compared with other spectroscopic analysis methods. The signal-to-noise (S/N) ratio in an NMR experiment is directly proportional to the number of spins. However, if the amount of the sample is limited, high sensitivity can be obtained by decreasing the coil size to increase the filling factor. The sensitivity of a solenoid coil, with a constant form factor (length/diameter), is inversely proportional to its diameter.^{1–3} The research using microcoil NMR has been expanded from high-resolution liquid-state NMR to solid-state NMR for materials science.^{4,5} However, application to organic compounds seems to be limited. In this study, a microcoil NMR probehead for solid-state NMR was prepared with two channels, ^1H (400 MHz) and X (from 75 to 165 MHz), targeting the standard analysis of organic compounds including biological samples under the cross-polarization (CP) condition. The performances are checked by reference samples, an application is shown for a biopolymer sample, silk fibroin, and then a structure and orientation study by using a small amount of silk fibers is shown. Compared to a conventional NMR probehead, this microcoil NMR probe that uses only 1/10 to 1/100 sample volume has a much bigger advantage for sample volume. Another advantage, especially for a fiber sample, is that distribution of the direction of the sample fibers can be analyzed by using a microcoil. Until now, in the NMR studies of fiber samples conventional probeheads with large size such as 10 mm Φ have been mainly used with CP for static samples. In such conventional probehead experiments,^{6–11} several thousands of threads for silk fiber sample should be prepared and glued. For this, this method has the problem that for spectral analysis it is very difficult to distinguish whether spectral change comes from the angle distribution of the fiber axis, or whether spectral change comes from the distribution of the thread direction that is coming from the misalignments of the sample threads from the internal distribution of the direction. On the other hand, the microcoil will have large advantage if the NMR spectrum can be observed by using very small number of

threads, and then only internal direction distribution of the fiber will be exactly analyzed. Therefore, this method can be very effective in analyzing the physical property of fibers associated with the degree of orientation.

From such a background, we have developed a microcoil probehead for solid-state NMR with a two-channel radio-frequency (rf) circuit, and we have obtained information on the distribution of the orientation of silk fibroin molecules in the fiber through ^{13}C observation with a proton-decoupling probehead.

Experimental Section

Probehead Design and Performance. The solenoid-shaped coil is the most typical and high performance coil for solid-state NMR, and in the case of the solenoid coil the sensitivity of probehead is inversely proportional to the diameter and length of the coil. This means that the signal from one spin is increased in a small coil. On the other hand, the number of spins is decreased by reduction of the coil size, which means losing sensitivity. In this work, application to biological materials is a target. For this, the probehead is made for ^{13}C (100 MHz)– ^1H (400 MHz) resonance. This requires some compromise about the size and volume of the coil. Compared to the previous microcoil work⁴ which used inorganic samples, carbon is not an ideal nucleus because the molecular weight of the sample of the biological materials is usually high and it also acts as a disadvantage even if we can use CP with protons. With such a background the probehead is designed for microgram samples for observation of carbon with proton decoupling and a coil of 1 mm (diameter) \times 5 mm (length) to achieve enough sensitivity even in macromolecules.

The probehead circuit is used with a conventional JEOL two-channel magic angle spinning (MAS) probehead by replacing the MAS stator and coil part of the original one. The goniometer of the coil to the B_0 field is realized by placing the coil, and it is checked by tracing the coil orientation to the protractor (Figure 1). The rf circuit is mainly used, but additional capacitors are added for tuning and matching for the microcoil. All NMR experiments are performed with a Varian-Chemmagetics Infinity400 NMR instrument operating at 400 MHz for ^1H . Static CP experiments are carried out with ramp–CP pulse sequence to avoid the mismatching of the Hartmann–Hahn condition

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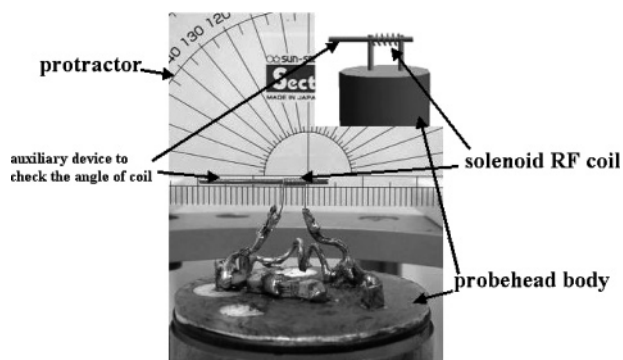


Figure 1. Photograph and illustration (top right) of the probehead including coil part and angle-adjusting devices (protractor and auxiliary rod for adjusting accurate angle of the coil). The angle can be set within $\pm 5^\circ$. The protractor and auxiliary device are removed during experiments.

caused by the B_1 inhomogeneity of the microcoil. An 83 kHz rf field is used for CP and proton decoupling to obtain spectra and the TPPM sequence is used during proton decoupling.

The performances are checked with CP experiments with adamantane and $[3\text{-}^{13}\text{C}]\text{alanine}$ with the following two aspects. One is that 1 mg of adamantane is used for checking the limit of the detection, with comparison of the microcoil probehead and a conventional probehead. The other is that the alanine sample is used for checking the sensitivity of the probehead from one spin by comparing the 1 mg sensitivity in a microcoil probehead and that in a conventional probehead.

NMR Observation of the Silk Fiber. A real application to biological materials with microcoil NMR is performed with $[1\text{-}^{13}\text{C}]\text{glycine}$ silk fibroin fibers from the *Bombyx mori* silkworm. The glycine residues are mainly in the crystalline region of silk fibroin, so it will be an acceptable probe for analysis of the crystalline region of the silk fibroin. The method for preparing the fibers is described elsewhere.^{8,9,11} From the ^{13}C CP/MAS spectra of the bulk sample, it was recognized that ca. 8% of glycine with 4% ^{13}C -labeled carbonyl carbon was ^{13}C -labeled in silk fibroin. A 70 μg oriented silk fiber sample with about 10 fibers of 1 cm length was used for the solid-state NMR. The sample of smaller and finer threads leads to better results for studying the distribution of the molecular orientation in the fiber, because the sample of a number of threads leads to the distribution of the fiber orientation. For this reason, about 10 threads are used in this experiment, and then they can be aligned manually and it is not necessary to glue the threads

such as the previous study¹¹ using a bulk sample. To compare with the results of the oriented fiber sample, an unoriented sample was also prepared. The NMR probehead is made of a solenoid microcoil with two channel tuning circuits in order to do ^{13}C (100 MHz)– ^1H (400 MHz) CP experiments in a 9.4 T magnet. The coil is settled at two angles to the external magnetic field (B_0). One is that the coil is perpendicular (90°) to B_0 . This means that the B_1 of the coil is also perpendicular to B_0 . The other is that the coil is tilted from B_0 and the angle between B_0 and B_1 is settled at 30° . When NMR observation is performed, the number of scans is 70 000 with a contact time of 2 ms and a recycle delay of 3 s. Thus, the total experiment time was 2 1/2 days.

Results and Discussion

Figure 2 shows the result for checking the performance of the microcoil probehead, where Figure 2a is checking the limit of detection and Figure 2b is checking the sensitivity of the probehead. Part a shows that the microcoil can observe a small amount of adamantane (1 mg) with 64 scans, and on the other hand a conventional 10 mm Φ probehead cannot observe it even with 1024 scans. In the performance of the limit of detection, the microcoil probehead has a greater advantage compared with conventional probeheads. The sensitivity test as shown in part b means that the S/N ratio for the microcoil probehead with a 1 mg sample was 44.2, and on the other hand the S/N ratio for a conventional 10 mm Φ probehead with a 242 mg sample was 1477.9. It is obvious that the signal intensity is increased with an increase in the sample volume. However, the absolute sensitivity as gained from nuclear spins can be estimated by the sensitivity per a unit volume or a unit weight. This means that the S/N ratio of the sample of 1 mg can be estimated to be 6.1 for the conventional probehead and thus the sensitivity of the microcoil probehead is about 7 times higher than that of the conventional probehead. It can be said that the microcoil NMR probehead can more effectively detect signals than the conventional probehead.

For the first biomaterial sample, static ^{13}C CP NMR spectra of silk $[1\text{-}^{13}\text{C}]\text{glycine}$ silk fibroin are observed as shown in Figure 3. The spectra of $[1\text{-}^{13}\text{C}]\text{glycine}$ in the fiber are mainly observed, but the natural abundance carbon peaks are also observed in the aliphatic region. It can be also estimated that the carbonyl peaks coming from natural abundance carbon are overlapped with those from $[1\text{-}^{13}\text{C}]\text{glycine}$, but the peak intensity is negligible because of broadening by chemical shift anisotropy.

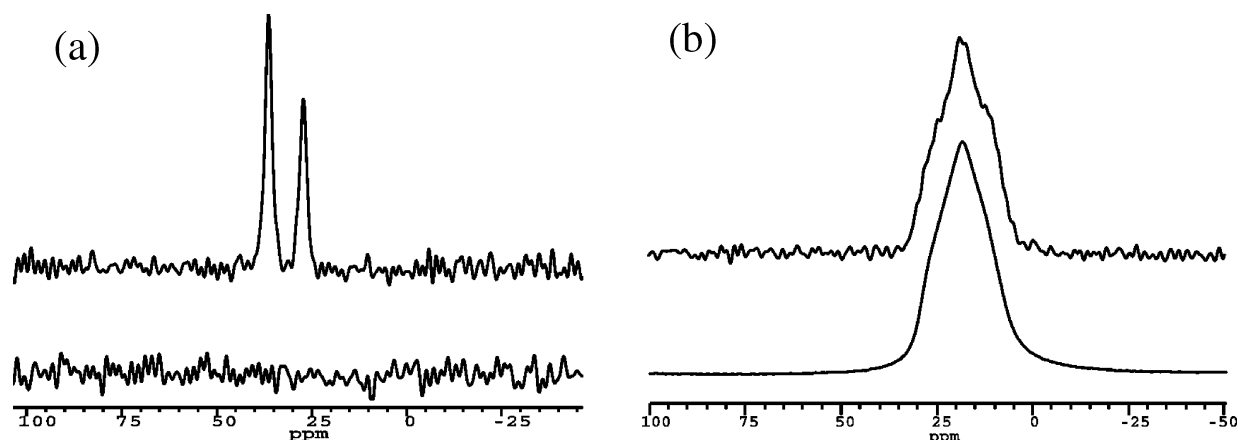


Figure 2. (a) Static CP spectra of 1 mg of adamantane with microcoil probehead (top) with 64 scans and conventional probehead (10 mm Φ) with 1024 scans (bottom). (b) Static CP spectra of $[3\text{-}^{13}\text{C}]\text{alanine}$ with a microcoil probehead (top) using 1 mg of sample and with a conventional probehead using 243 mg of sample (bottom).

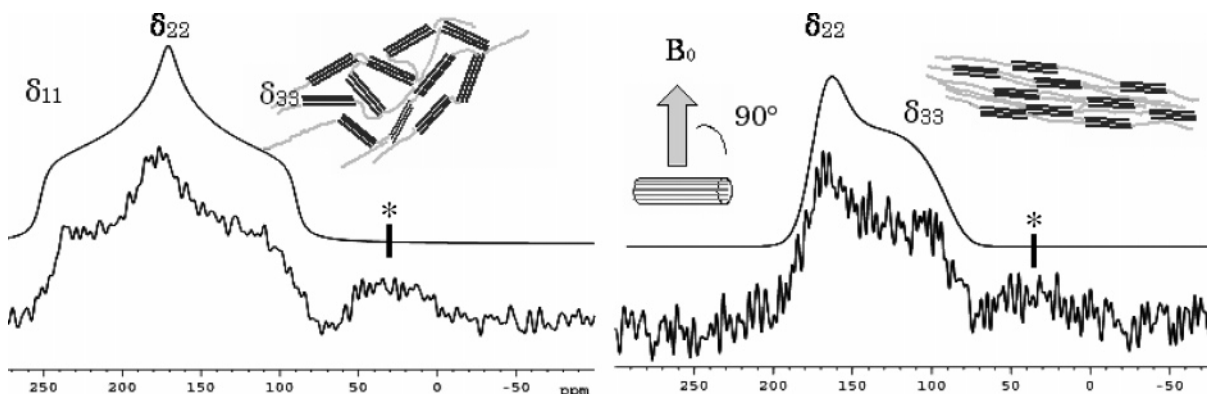


Figure 3. ^{13}C CP static spectra and simulated spectra of silk fibroin: (left) unoriented sample observed with conventional probehead; (right) oriented fiber sample ($70\ \mu\text{g}$) observed with microcoil probehead. The sample is placed perpendicular to the magnetic field (90°). Superposed spectra are fitted spectra of it. The peaks marked with asterisks (*) come from natural abundance carbon.

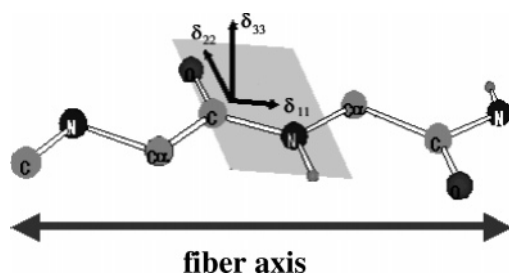


Figure 4. Representation of fiber axis, molecular axis, and directions of chemical shift tensor components in the silk fiber.

The spectra have a reasonable S/N ratio, enough to analyze the structure. The left-side spectrum comes from unoriented sample and the right-side spectrum comes from the oriented fiber sample placed perpendicular to B_0 as shown together with the simulated spectrum. The lower field signal comes mainly from $[1-^{13}\text{C}]$ -glycine and the small peak from 0 to 50 ppm comes from natural abundance signals. The unoriented sample gives a broad peak which can fit a powder spectrum. From the simulated spectrum, the chemical shift tensor components are $\delta_{11} = 245\ \text{ppm}$ ($\pm 5\ \text{ppm}$), $\delta_{22} = 180\ \text{ppm}$ ($\pm 5\ \text{ppm}$), and $\delta_{33} = 88\ \text{ppm}$ ($\pm 5\ \text{ppm}$). The fitted spectrum is a typical powder pattern, so the effect of ^{13}C – ^{13}C dipolar coupling caused by labeling of the sample is limited. These values agree with those reported previously.⁹ The oriented fiber sample can be also fitted as shown with the distribution factor¹⁰ of 15. It gives δ_{22} and δ_{33} , but δ_{11} is missing. The reason comes from the direction of the chemical shift tensor component δ_{11} in the fibroin. According to the results reported previously,⁷ the chemical shift tensor component of δ_{11} is along the fiber axis, the δ_{22} is along the direction of the carbonyl bond, and the δ_{33} is perpendicular to both axes. The directions of the chemical shift tensor components of the carbonyl glycine in the fiber are shown in Figure 4. The δ_{11} is parallel to B_1 (perpendicular to B_0), and thus this component is missing from the spectrum. This is the reason only δ_{22} and δ_{33} can be observed in the fiber spectrum. The result of a microgram sample served with this probehead agrees with that of bulk sample.⁷ The solenoid coil is one of the best shapes for solid-state NMR coils, but the direction of the B_1 field is generated along the long direction. For the microcoil probehead, the sample silk fibroin fiber can be placed along B_1 , and δ_{22} and δ_{33} can be observed if the coil is placed perpendicular to B_0 ; these two chemical shift tensor components will be decreased and δ_{11} is increased if the angle between the fiber axis and B_0 is decreased.

Figure 5 shows the spectrum of the same sample in which the angle between the fiber axis and B_0 is 30° . The spectrum with an intense signal corresponding to δ_{11} and weak signal

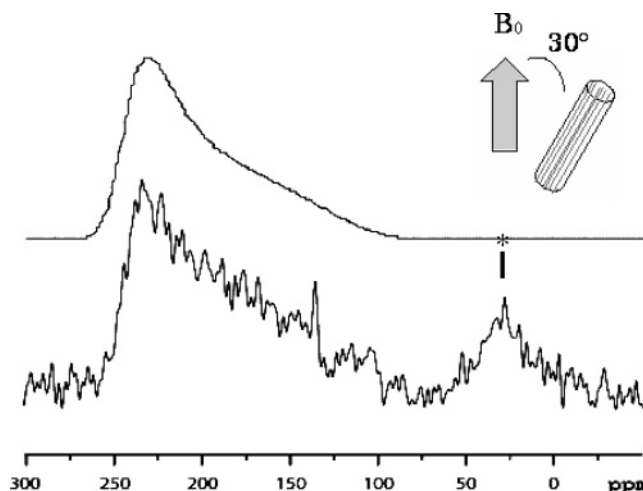


Figure 5. Observed static ^{13}C CP spectra of oriented fiber sample of silk fibroin ($70\ \mu\text{g}$) observed with a microcoil probehead together with simulated spectrum. The sample is placed at 30° from the external magnetic field (B_0). The peak marked with an asterisk comes from natural abundance carbon.

corresponding to δ_{22} and δ_{33} is shown together with a simulated spectrum with a distribution factor of 15. Compared to the 90° spectrum in which the angle between the fiber axis and B_0 is 90° , the chemical shift range is large because all of the chemical shift tensor components are observed. The 0° spectrum in which the angle between the fiber axis and B_0 is 0° will be the best for silk fibroin sample to discuss the distribution of the orientation because only δ_{11} is observed and the distribution factor is small. The solenoid-shaped coil used in this work is one of the best shapes of coils to obtain a reasonable S/N ratio and homogeneity of B_1 . The fiber sample is settled along the coil to avoid the distribution of orientation, so the coil cannot settle 0° to B_0 because B_1 will be parallel to B_0 and no NMR signal can be obtained. A saddle-type coil is an alternative shape, but it was tested with silk sample and it was not suitable for S/N and B_1 homogeneity. Another shape of coil is necessary. Recently, van Bantum et al.⁵ introduced the planar strip line shape for generating the B_1 field; this might have a high performance in S/N and also in the homogeneity of B_1 .

Figure 6 shows the simulated spectra by changing the distribution factor of the chemical shift tensor components,¹¹ and the best-fit result of the spectra obtained by the microcoil probehead and also the conventional probehead. The distribution factor used in here is defined by Yeo et al.¹⁰ with the Gaussian distribution of the angle. The number p is the width of the half-height of the Gaussian distribution. Simulation with the

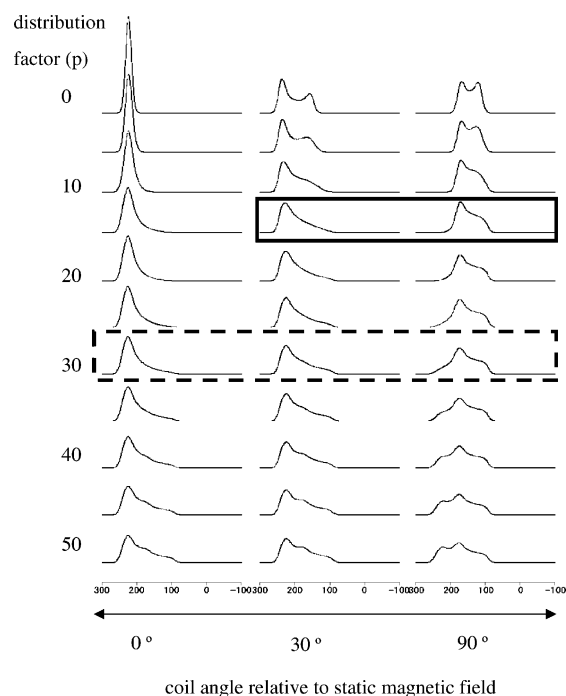


Figure 6. Simulated ^{13}C spectra of $[1-^{13}\text{C}]\text{Gly}$ carbon of silk fibroin using experimental data reported previously¹¹ by changing the distribution factor and the angle of coil. The spectra in the dashed-line box show the best-fit result of bulk sample,¹¹ and the spectra in the solid-line box show the best-fit result with microcoil sample.

“distribution factor” in the spectrum is needed for the following two reasons. The first comes from the distribution of the orientation of the chain of molecules. This will be a favorable parameter for structural analysis of the fibers because if the molecules in fiber are aligned well, the fiber will have a low modulus. Therefore, the result obtained from the observed NMR spectra may be an index of the toughness of the fiber. The second comes from the preparation of the sample. The bulk sample cited from Demura et al.¹¹ is prepared by gluing the fibers to make the big sample. It will have some distribution in orientation in the glued sample. Because the sample with the microcoil uses a much smaller amount of fibers, the distribution in orientation can be expected to be smaller than that of the bulk sample. This is shown in Figure 6. The spectra in the solid-line box are best-fit data for the microcoil result, and the spectra in the dashed-line box are the data from the bulk sample. This shows that the bulk sample has a larger distribution factor. The origins of both samples are the same. The preparation of the samples plays an important role. The bulk sample tends to have

a large distribution of orientation because it a large amount of samples have to be glued. On the other hand, the sample used for the microcoil is easily picked up; the sample is easily fit in the coil and it is not necessary to glue the sample to force the sample to align. This will cause the small distribution of the orientation of fibers and make a smaller distribution in the spectra. To investigate the distribution of the molecule in the fiber, the error caused by sample preparation should be as small as possible. Therefore, it can be said that the distribution factor obtained by the microcoil is more precise compared with that from the conventional probehead, and thus that microcoil NMR is a better tool for these analyses.

Conclusion

It can be concluded that even a microgram sample of silk fiber can be observed with a 400 MHz NMR spectrometer with a microcoil NMR probehead. The performance of the sensitivity is enough for NMR study for biological samples. One of the examples is shown for silk fiber with a microgram sample 90° and 30° from the magnetic field. The microcoil NMR probehead can be easily applied to analysis of other fibers for orientation and the orientation distribution of the molecules in fibers.

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