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Peptide Nanoassemblies

Towards Structure Determination of Self-Assembled Peptides Using Dynamic Nuclear Polarization Enhanced Solid-State NMR Spectroscopy**

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Bio-inspired self-assemblies made of peptide building blocks have great potential for nanotechnology ranging from biological and pharmaceutical applications to (opto)electronics.[1–3] With these goals, a variety of peptide nanoassemblies have been studied and designed over the last few decades.[4] Inevitably, structural studies at an atomic scale are crucial to unravel the mechanisms that drive nanoassembly formation as well as to relate these structures to their physical and chemical properties. However, structure determination at an atomic level is challenging essentially because of the difficulty associated with using X-ray crystallography on such nanoassemblies.[3]

Solid-state NMR (SSNMR) spectroscopy is a powerful and promising technique for structural analysis of nanoassemblies. In principle, SSNMR spectroscopy can be used for any form of solid sample from well-ordered crystals to disordered powders.[5] Furthermore, the recent development of high-field magic-angle-spinning dynamic nuclear polarization (MAS-DNP) allows one to compensate the inherent low sensitivity of NMR experiments.[6]

The main concern regarding the DNP technique is the relative line broadening typically encountered in biomolecular systems at low temperatures (LT).[7] This broadening is induced by a change of dynamics at LT which leads to the detection of conformational disorder. This drawback can be circumvented when MAS-DNP is applied to crystalline materials that keep their ordered structures and yield narrow linewidths even at LT.[8–10] Therefore, they are a priori favorable for DNP experiments. In fact, a natural-abundance 2D ^{13}C–^{13}C correlation experiment using matrix-free (MF) sample preparation with an experimental time of only tens of minutes has recently been reported on crystalline cellulose.[9] This illustrated the realistic feasibility of studying atomic-scale structures of unlabeled organic systems with NMR spectroscopy. Since self-assembling systems possess ordered structures, they are also a priori suitable candidates for DNP experiments.

Herein, we demonstrate the possibility of structural study on challenging nanoassemblies. In particular, we chose to tackle systems based on the diphenylalanine (FF) dipeptide[2, 11–13] which are currently emerging as a new class of organic semiconductors.[14,15] FF is also a core motif of Alzheimer's amyloid-β and plays a key role in the self-assembly of the amyloid.[16] Amongst FF derivatives, we particularly focus on the cyclic form of FF (cyclo-FF). Recent articles by Adler-Abramovich et al.[17] and Lee et al.[18] reported that FF can be self-assembled into cyclo-FF-based nanotubes (NTs)/nanowires (NWs) prepared by a vapor-phase transport method.

Here, we introduce an efficient method to prepare self-assembled peptide NTs suitable for MF-DNP measurements.[9] We perform 2D ^{13}C–^{13}C correlation experiments on cyclo-FF NTs that provide fundamental structural information such as hydrogen-bonding and π-stacking interactions. Furthermore, the naturally low isotopic abundance allows one to detect both intra- and intermolecular long-range interactions during dipolar mediated polarization transfer experiments. This is the first and major step in determining de novo 3D structures of nanoassemblies.

For DNP experiments, polarizing agents need to be distributed uniformly in a sample. Thus, it is highly ineffective to add them after NT formation because of rapid aggregation of NTs (see Section S2 in the Supporting Information). Alternatively, we used a solution-based method to obtain cyclo-FF NTs with a uniform distribution of polarizing agents. This sample preparation technique is original and extremely useful for "DNP-ready" samples, especially self-assembling systems that tend to aggregate. Adding anti-solvents (water and methanol in this case) to a cyclo-FF solution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) creates NTs that aggregate immediately after their formation. Here, the polarizing agent, TOTAPOL,[19] was added simultaneously with the anti-solvents so that TOTAPOL biradicals were trapped inside the NTs when they formed and outside the NT walls when aggregation occurred. Details of the procedure are described in the Experimental Section.

Formation of cyclo-FF NTs was confirmed by scanning electron microscopy (SEM; Figure 1). The diameters of the obtained NTs are not uniform but typically fall in the range of

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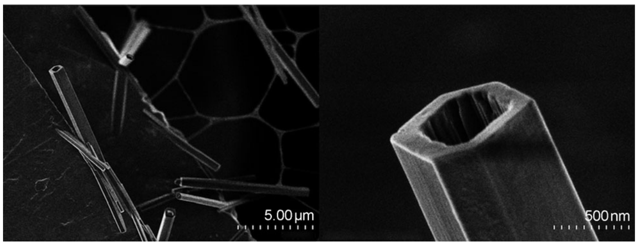

Figure 1. SEM images of the cyclo-FF NTs prepared by a solution-based method. Image of NTs (left) and a magnified image of one of the NTs from the left image (right).

100–1000 nm. The NTs have wall thicknesses smaller than 200 nm. This is thin enough to perform efficient DNP experiments.[8] Larger objects require more time for DNP build-up and thus need to possess a long spin-lattice relaxation time constant (T_1) for substantial polarization transfer.[10]

A DNP-enhanced 1D ^{13}C-CPMAS spectrum is shown in Figure 2 and compared to that obtained by conventional SSNMR. A DNP enhancement factor ($\varepsilon_{\mathrm{DNP}}$) of 8.8 was observed (by comparing microwaves-on and -off spectra). No line-broadening is observed at LT, since the cyclo-FF molecules are well-ordered and rigid, which prevents any conformational distribution in the frozen state. Furthermore, since we utilized the MF approach, there is no deleterious dilution effect of the sample of interest unlike other sample preparation methods.

The T_1 of ^{1}H spins in cyclo-FF is exceedingly long because of its rigidity ($T_1 > 10$ minutes at room temperature). Therefore it is infeasible to perform 2D ^{13}C–^{13}C correlation experiments even on a ^{13}C-enriched system. On the other hand, in the DNP sample where radicals are uniformly distributed, the T_1 of ^{1}H spins is shortened to 2.6 s because of the presence of paramagnetic spins. This alone is already leading to a substantial time-saving factor greater than 230 for SSNMR measurements.

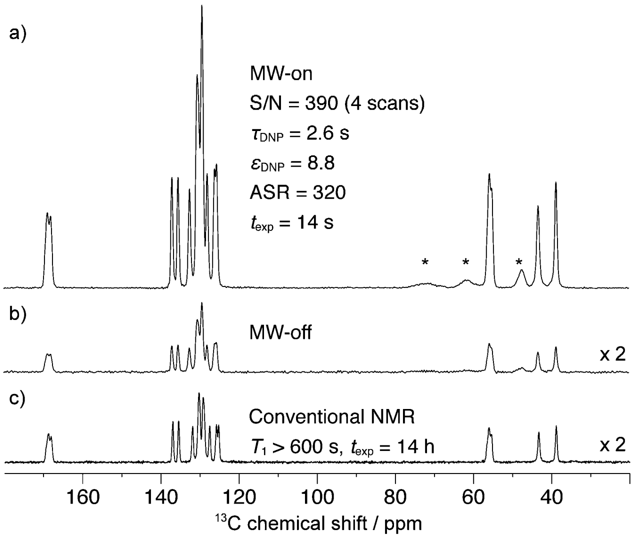

Figure 2. ^{13}C-CPMAS spectra of natural-abundance cyclo-FF NTs recorded using a DNP equipped spectrometer with a) microwaves-on and b) -off at 105 K and 9.4 T (3.2 mm zirconia rotor) and using c) a conventional NMR spectrometer at room temperature and 9.4 T (4 mm zirconia rotor). The MAS frequency was 13 kHz. Experimental times were 14 s (a recycle delay of 3.4 s, 4 scans) and 14 h (a recycle delay of 300 s, 168 scans) for the DNP experiments and the conventional NMR experiment, respectively. Asterisks indicate the signals from glucose, glycerol, and remaining methanol.

Signal-to-noise ratios (S/N) of DNP-enhanced NMR and conventional NMR spectroscopy were 390 with 4 scans and a recycle delay of 3.4 s (S/N = 562 at $5T_1$) and 73 with 168 scans and a recycle delay of 300 s, respectively. Overall, an absolute sensitivity ratio (ASR),[9] determined by comparing the S/N per unit time of DNP-enhanced NMR and conventional NMR spectroscopy, of 320 was found. This corresponds to a remarkable time-saving factor of 10^5. The ASR represents the "real" gain of performing DNP and includes positive contributions (DNP enhancement factor, Boltzmann factor (including thermal noise), reduced repetition rate) as well as negative contributions such as signal bleaching, changes in linewidths and reduced sample volumes. The negative contributions from the latter two are minimized using our approach. Note that a 4 mm rotor (80 μL) was used for conventional NMR spectroscopy whereas a 3.2 mm rotor (42 μL) was used for the DNP experiments.

The S/N per unit time returned from the DNP experiments makes it possible to perform 2D ^{13}C–^{13}C correlation experiments without ^{13}C enrichment. Thus, one such through-bond experiment[20] was recorded at natural abundance on the cyclo-FF NTs (Figure 3). All one-bond correlations were observed within 5 h. It is worth noting that no ^{13}C–^{13}C correlations were seen in a solution-state NMR correlation experiment recorded in 60 h even though the amount of sample used was 1.5 times larger than that for DNP-enhanced SSNMR spectroscopy.

All signals for the cyclo-FF NTs were assigned and compared to the chemical shifts of cyclo-FF molecules dissolved in HFIP and obtained by solution-state NMR spectroscopy (Table 1). The two phenylalanine residues show the same chemical shifts in solution indicating that the chemical environments are identical for both residues. This is no more the case in the solid state because of the absence of molecular motions. Most of the ^{13}C resonances show significant deviations compared to solution-state NMR data; this being a clear signature of intra- and intermolecular interactions. For instance, $C^{\delta 1}$ of Phe 2 (δ_{21}) experiences a downfield shift indicating a π–π interaction between the aromatic ring of Phe 1 and δ_{21} (T-shaped interaction). Furthermore, the narrow linewidths of the aromatic peaks at LT suggest the presence of multiple π–π interactions that can prevent ring flips. Upfield shifts on the carbonyl and alpha carbons (compared to empirical values in proteins) suggest that cyclo-FF forms β-sheet-type hydrogen bonds with neighboring molecules. These interactions are fully consistent with the model of cyclo-FF NWs obtained by Lee et al. using X-ray powder diffraction.[18]

To further demonstrate the feasibility of supramolecular structure determination using this approach, through-space ^{13}C–^{13}C dipolar correlation experiments were performed using SPC5 recoupling[21] at various mixing times (Figure S6).

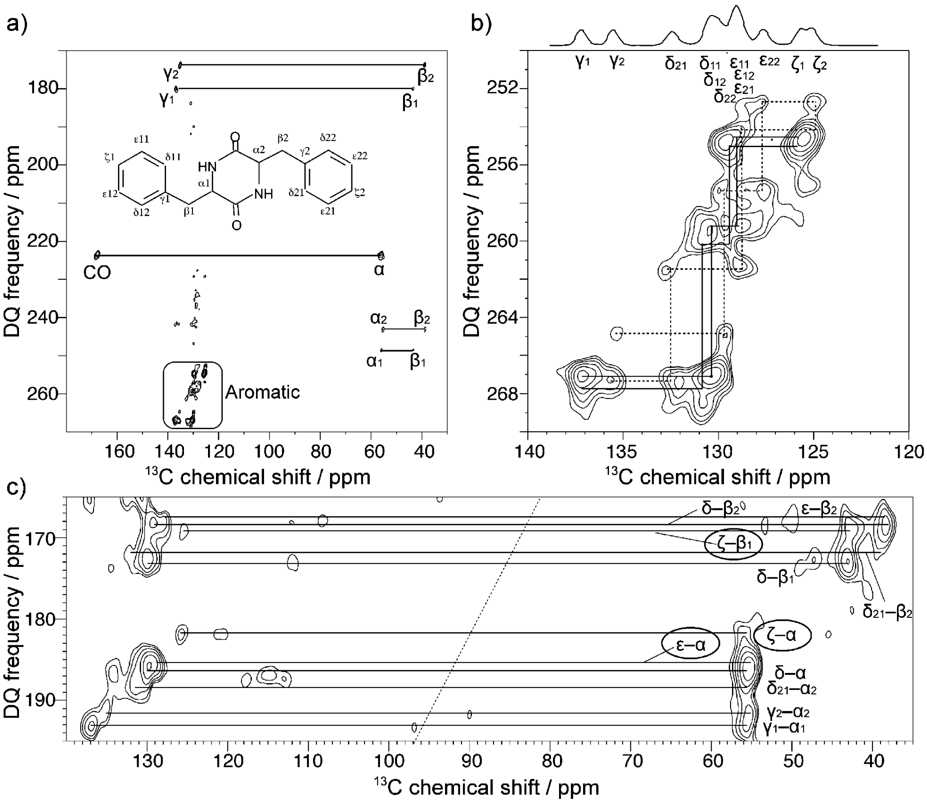

Figure 3. a) A DNP-enhanced ^{13}C–^{13}C 2D refocused INADEQUATE[20] spectrum of natural-abundance cyclo-FF NTs recorded at 105 K. The spectrum was obtained in 4.8 h with a recycle delay of 1 s and 16 ms (in total) of echo periods at a MAS frequency of 12.5 kHz. The C^{α}–C^{β} cross-peaks are folded and appear between 240–250 ppm in the DQ frequency dimension. b) The aromatic region was enlarged to show all correlations within the aromatic rings. An external reference (1D ^{13}C-CPMAS) is shown on the top. c) A DNP-enhanced 2D DQ-SQ ^{13}C–^{13}C dipolar correlation spectrum. The MAS frequency was 10.5 kHz and the mixing time was set to 7.62 ms. For other experimental conditions, see Section S4 in the Supporting Information. DQ: double quantum, SQ: single quantum.

Owing to the spin dilution (only 1.1 % of the carbon nuclei are ^{13}C spins), the spin dynamics involved during ^{13}C–^{13}C polarization transfer are much simpler than in the case of a uniformly labeled system where strong dipolar couplings dominate and strongly quench long-distance transfer (dipolar truncation). As shown in Figure S6, short mixing times (1.5 ms) favor mostly one-bond correlations (1.5 Å) whereas longer mixing (4.6 ms) clearly yields two-bond (2.5 Å) and three-bond (2.7 Å for the cis- and 3.8 Å for the trans-conformer) correlations. For even longer mixing times (7.6 ms), multiple long distance contacts can clearly be identified (Figure 3c). Among them the C^{α}–C^{ε}, C^{α}–C^{ζ}, and C^{β}–C^{ζ} cross-peaks (indicated by circles) mainly result from intermolecular contacts (for details, see Section S4 in the Supporting Information). These results clearly pave the way towards supramolecular structure determination.

In conclusion, we have shown a novel sample preparation method targeting DNP experiments on self-assembling peptides. Using this approach, polarizing agents can be uniformly added even into samples that inherently tend to strongly aggregate. Combining this approach and the MF method enabled us to perform 2D ^{13}C–^{13}C correlation experiments on an unlabeled self-assembled peptide. All signals were assigned and supramolecular structural information such as hydrogen bonding and π–π stacking was obtained. Furthermore, the feasibility of detection of intermolecular contacts was demonstrated.

Self-assembling supramolecular systems exhibiting narrow linewidths even at LT proved to be particularly pertinent for DNP experiments (especially with the MF approach). As such, DNP-enhanced SSNMR spectroscopy is a perfect tool for de novo structural determination of nanoassemblies that are unattainable using X-ray crystallography. Finally, it is important to note that this work is useful not only for unlabeled nanoassemblies but also has a great impact on self-assemblies that consist of larger and/or more complex molecules such as amyloid fibrils which are usually ^{13}C- and/or ^{15}N-enriched for SSNMR studies. Signal overlapping is a major issue in such systems. The results demonstrated here will facilitate higher-dimensional NMR experiments that lead to separation of overlapped signals. This study exemplifies that DNP-enhanced SSNMR spectroscopy has the potential to become a key

Table 1: Chemical shifts (ppm) of cyclo-FF NTs obtained by DNP-enhanced SSNMR and cyclo-FF monomers obtained by solution-state NMR spectroscopy.

	CO	C^{α}	C^{β}	C^{γ}	$C^{\delta1}$	$C^{\delta2}$	$C^{\varepsilon1}$	$C^{\varepsilon2}$	C^{ζ}
Phe 1[a]	168.8	55.7	43.3	137.0	130.8	130.4	129.5	129.1	126.0
Phe 2[b]	168.0	55.2	38.7	135.4	132.4	130.0	128.8	127.9	125.5
Monomer[c]	168.8	56.5	39.5	134.1	129.8	129.8	129.2	129.2	128.0

[a,b] Phenylalanine 1 (a) and 2 (b) of cyclo-FF NTs measured by DNP-enhanced SSNMR spectroscopy. Chemical shifts were calibrated indirectly through the carbonyl peak of glycine (176.03 ppm relative to TMS) at room temperature. [c] The cyclo-FF monomer was dissolved in HFIP and measured by solution-state NMR spectroscopy using TMS as an external standard. The assignment of the chemical shifts was performed using empirical data.

technique for structure determination of self-assembled (bio)materials.

Experimental Section

Sample preparation: Cyclo-FF powder (20 mg) purchased from Bachem (Weil am Rhein, Germany) was dissolved in 750 μL of HFIP. Deuterated methanol (0.5 mL) and D_2O (2.5 mL) containing the TOTAPOL biradical (0.16 mg), glucose (0.5 mg), and [D_8]glycerol (2 mg) were added to the HFIP solution. Immediately, cyclo-FF NTs were formed and precipitated. The solution was placed on a Petri dish and dried under vacuum. Since moisterization of a sample is needed for obtaining good DNP enhancement,[9] glucose and glycerol were added to retain some moisture. This preparation was repeated once more to fill up a thin-walled 3.2 mm zirconia rotor.

NMR experiments: All experiments were performed on a Bruker AVANCE III 400 MHz wide-bore NMR system equipped with a 263 GHz gyrotron, a transmission line, and a low-temperature capable (about 100 K) triple resonance 3.2 mm MAS probe. Details are described in Section S1 in the Supporting Information.

SEM measurements: The NTs were coated with carbon and the images were taken using a HITACHI S-5500 scanning electron microscope.

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