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

Hiroki Takahashi, Bastien Viverge, Daniel Lee, Patrice Rannou, and Gaël De Paëpe*

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 very challenging essentially due to the difficulty associated with using X-ray crystallography on such nanoassemblies.^[3]

Solid-state NMR (SSNMR) is a powerful and promising technique for structural analysis of nanoassemblies. In principle, SSNMR 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 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 ¹³C–¹³C 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. Since self-assembling systems possess ordered structures, they are also *a priori* suitable candidates for DNP experiments.

In this contribution, 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 semiconductor.^[14,15] FF is also a core motif of Alzheimer's β -amyloid and plays a key role in the amyloid's self-assembly.^[16] Amongst FF derivatives, we particularly focus on the cyclic form of FF (cyclo-FF). Recent literature by Adler-Abramovich *et al.* and Lee *et al.* reported that FF can be self-assembled into cyclo-FF-based nanotubes (NTs)/nanowires (NWs) prepared by a vapor phase transport method.^[17,18]

In this work, we introduce an efficient method to prepare self-assembled peptide NTs suitable for MF-DNP measurements.^[9] We perform 2D ¹³C–¹³C 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 inter-molecular 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 due to rapid aggregation of NTs (Supporting Information, S2). 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 Experimental Section.

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author. ((Please delete if not appropriate))

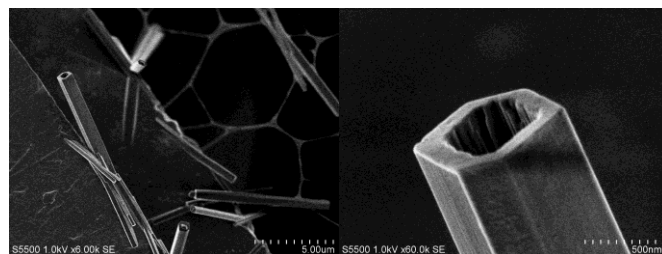


Figure 1. SEM images of the cyclo-FF NTs prepared by a solution-based method. Image of NTs (left) and a magnified image of one of the NTs from the left image (right).

Formation of cyclo-FF NTs was confirmed by scanning electron microscopy (SEM) (Fig. 1). The diameters of the obtained NTs are not uniform but typically fall in the range of 100 – 1000 nm. The NTs have wall thicknesses of < 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 T_1 for substantial polarization transfer.^[10]

A DNP-enhanced 1D ^{13}C -CPMAS spectrum is shown in Fig. 2 and compared to that obtained by conventional SSNMR. A DNP enhancement factor (ϵ_{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.

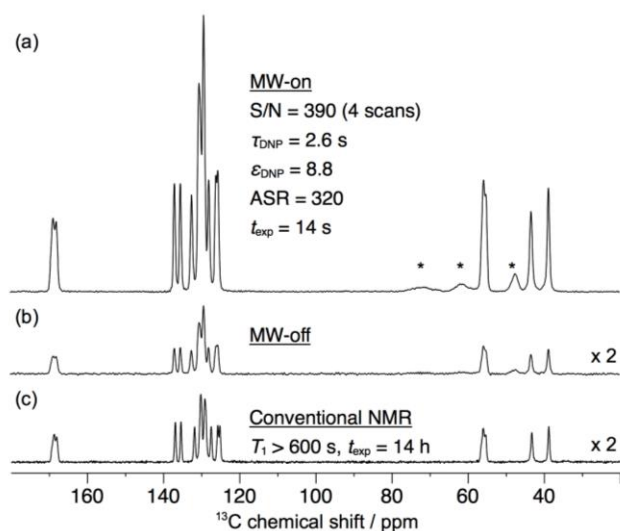


Figure 2. ^{13}C -CPMAS spectra of natural-abundance cyclo-FF NTs recorded using a DNP-equipped spectrometer with microwaves-on (a) and -off (b) at 105 K and 9.4 T (3.2-mm zirconia rotor) and using a conventional NMR spectrometer at room temperature and 9.4 T (4-mm zirconia rotor) (c). The MAS frequency was 13 kHz. Experimental times were 14 s (a recycle delay of 3.4 s, 4 scans) and 14 hours (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.

The spin-lattice relaxation time constant (T_1) of ^1H spins in cyclo-FF is exceedingly long due to 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 ^1H spins is shortened to 2.6 s due to the presence of paramagnetic spins. This alone is already leading to a substantial time-saving factor of > 230 for SSNMR measurements.

Signal-to-noise ratios (S/N) of DNP-enhanced NMR and conventional NMR 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, 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 the conventional NMR 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 enables one 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 (Fig. 3). All one-bond correlations were observed within 5 hours. It is worth noting that no ^{13}C - ^{13}C correlations were seen in a solution-state correlation experiment recorded in 60 hours even though the amount of sample used for solution-state NMR was 1.5 times larger than that for DNP-enhanced SSNMR.

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 (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 due to 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 inter-molecular interactions. For instance, $\text{C}^{\delta 1}$ 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 bonding 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 (Fig. S6). 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 Fig. S6, short mixing times (1.5 ms) favor mostly one-bond correlations (1.5 \AA) whereas longer mixing (4.6 ms) clearly yields two-bond (2.5 \AA) and three-bond (2.7 \AA for *cis*, 3.8 \AA for *trans*) correlations. For even longer mixing times (7.6 ms), multiple long distance contacts can clearly be identified (Fig. 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 inter-molecular contacts (for details, see Supporting Information, S4). These results clearly pave the way towards supramolecular structure determination.

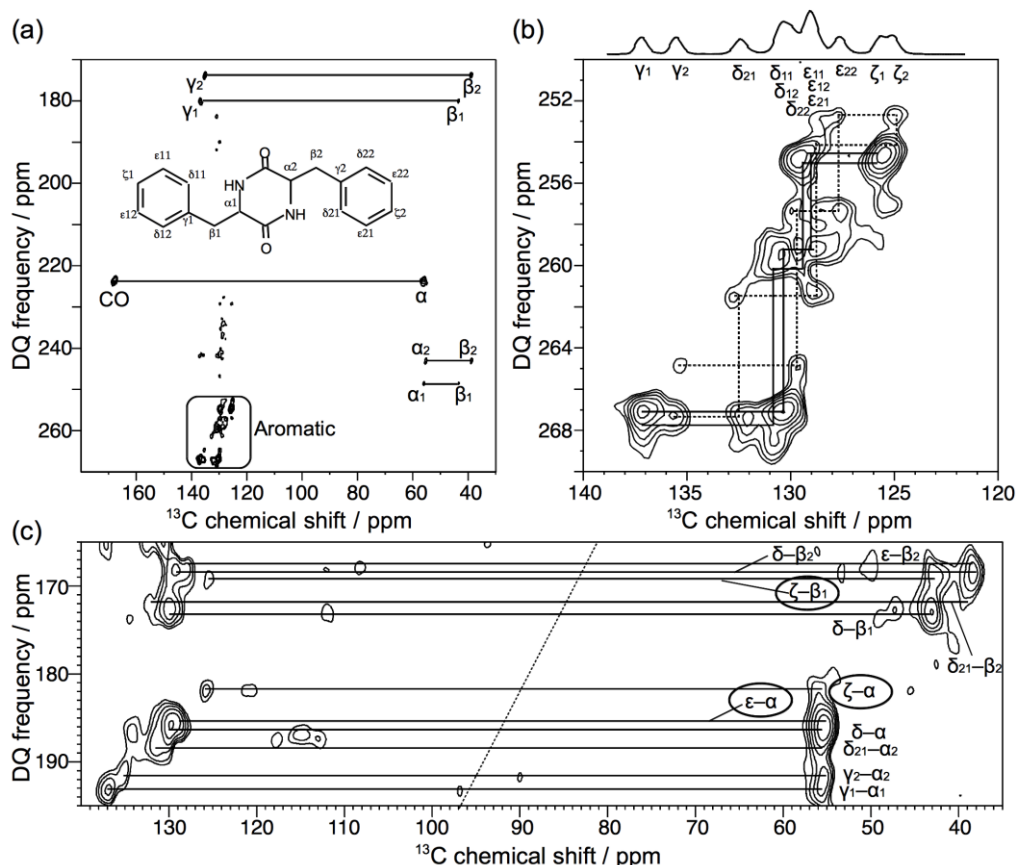


Figure 3. a) A DNP-enhanced ¹³C-¹³C 2D refocused INADEQUATE^[20] spectrum of natural-abundance cyclo-FF NTs recorded at 105 K. The spectrum was obtained in 4.8 hours with a recycle delay of 1 s and 16 ms (in total) of echo periods at a MAS frequency of 12.5 kHz. C^α-C^β cross peaks are folded and appear 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 ¹³C-CPMAS) is shown on the top. c) A DNP-enhanced 2D DQ-SQ ¹³C-¹³C 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 Supporting Information, S4. DQ: double quantum, SQ: single quantum.

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 ¹³C-¹³C 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 inter-molecular 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 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 ¹³C and/or ¹⁵N-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 has the potential to become a key technique for structure determination of self-assembled (bio)materials.

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

	CO	C ^α	C ^β	C ^γ	C ^{δ1}	C ^{δ2}	C ^{ε1}	C ^{ε2}	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. Chemical shifts were calibrated indirectly through the carbonyl peak of glycine (176.03 ppm relative to TMS) at room temperature. [c] Cyclo-FF monomer was dissolved in HFIP and measured by solution-state NMR using TMS as an external standard. Chemical shifts assignment was performed using empirical data.

Experimental Section

Sample preparation: 20 mg of cyclo-FF powder purchased from Bachem (Weil am Rhein, Germany) was dissolved in 750 μ L of HFIP. 0.5 mL of deuterated methanol and 2.5 mL of D₂O containing 0.16 mg of TOTAPOL biradical, 0.5 mg glucose and 2 mg [D₆]glycerol 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 moisturation of a sample is needed for obtaining good DNP enhancement,^[9] glucose and glycerol were added to help 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 (~100 K) triple resonance 3.2 mm MAS probe. Details are described in Supporting Information, S1.

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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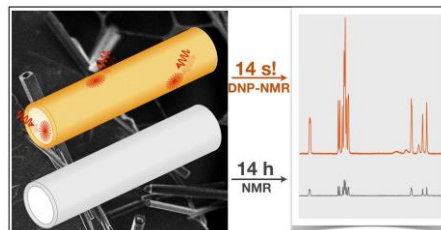
Entry for the Table of Contents (Please choose one layout)

Layout 2:

Peptide Nanoassemblies

Hiroki Takahashi, Bastien Viverge,
Daniel Lee, Patrice Rannou, and Gaël
De Paëpe* _____ Page – Page

Towards Structure Determination of
Self-Assembled Peptides using
Dynamic Nuclear Polarization Enhanced
Solid-State NMR



Dynamic nuclear polarization (DNP) enhanced solid-state NMR was performed on self-assembled peptide nanotubes prepared with a new 'DNP-friendly' protocol. This approach yields significant experimental time-savings (about 5 orders of magnitude). This was used to exemplify the feasibility of supramolecular structural study of organic nanoassemblies at an atomic scale using DNP-enhanced solid-state NMR.