Solid-State NMR Analysis of Biomolecules: New BioSolids Experiments for VnmrJ 3.2 and BioPack

Technical Overview

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Introduction

Structural biologists and biochemists rely on NMR analysis for the determination of protein structures in solution, and increasingly for membrane, crystalline, and other solid-state protein structures. Remarkable advances in solid-state NMR (SSNMR) and magic-angle spinning (MAS) techniques during the past few years are now contributing significantly to the characterization of protein structure and function. SSNMR approaches make possible the direct study of membrane proteins and other large or insoluble biological structures that are difficult to study by solution-state NMR, while complementing X-ray crystallographic methods. A substantial body of solid-state experiments now exists among Agilent NMR users for the study of ^{13}C, ^{15}N, and other isotope-enriched proteins. These SSNMR experiments are similar in purpose and scope to those traditionally used for the analysis of molecules in solution, such as those found in the Agilent BioPack software package. This technical overview describes the new Agilent BioSolids Pack which is a repository for SSNMR sequences designed to investigate protein samples.

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The Advantages of Biomolecular MAS SSNMR

In spite of the remarkable success of structural biology for the analysis of soluble proteins over the last three decades, membrane proteins still remain elusive partly because of the difficulty in finding a well-behaved membrane mimetic environment. Membrane proteins are major drug targets,[6] however they currently represent < 2 % of known protein structures. This is because traditional, solution-state NMR studies usually render membrane proteins soluble using detergents. NMR studies of membrane protein-detergent complexes have a very low success rate because they tumble slowly in solution, resulting in increased relaxation rates that make it very challenging to obtain NMR spectra with high resolution and good signal-to-noise ratios. In addition, there is increasing evidence that lipid species play an important structural and functional role.[6] Therefore, studying the membrane protein in its native membrane environment provides the most biologically relevant information.

Magic-angle spinning SSNMR (MAS SSNMR) is a very rapidly developing field which includes significant tools for the study of protein structure and function. MAS SSNMR offers a clear advantage because it enables the study of membrane proteins in the lipid bilayer and in other solid-state contexts. Since the earliest structural assignments of crystalline proteins,[1, 2] MAS SSNMR has undergone a revolution in pulse sequence development, assignment techniques, and protein structure determination. Many laboratories are now engaged in probing conformational dynamics and ligand binding, and they are solving the complete structures of biological macromolecules using solid-state methods based on magic-angle spinning.

MAS SSNMR can be used to study proteins in a wide range of situations, including protein micro-crystals, aggregates, and native membrane-bound proteins *in situ*. In theory, protein size is not a limiting factor with MAS SSNMR but large proteins are more difficult to study because of the current limitations in spectral sensitivity and dispersion. MAS SSNMR techniques have also been used effectively to study protein aggregates associated with neurodegenerative diseases, such as amyloid fibril formation in Alzheimer's disease[3] and alpha-synuclein[4, 5] as it relates to Parkinson's disease. Since MAS SSNMR can resolve structural issues in protein aggregates and membrane proteins it works where other high-resolution biophysical techniques, such as X-ray crystallography and solution NMR, struggle or fail.

The New Biomolecular Solid-State Software

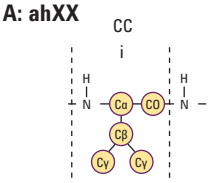
This initial release of BioSolids sequences for VnmrJ 3.2 provides a foundation for ^{13}C-detection, and 2D and 3D experiments that correlate ^{13}C and ^{15}N chemical shifts (Table1), as well as a set of 1D calibration methods. Additional sequences will be incorporated as they become available. The current experiments are provided through multifunctional pulse sequences, each associated with a particular 2D or 3D polarization-transfer pathway. The user can select from a variety of mixing sequences and evolution methods as options through parameter flags. The evolution options include a simple delay with hetero-J refocusing, constant-time evolution, or evolution with band-selective refocusing. All sequences allow for constant-time decoupling and optional Y-channel (^{15}N) decoupling. They also have the potential to be used with ^{1}H, ^{19}F, and ^{13}C.

These sequences are available for VnmrJ 3.2 and are programmed using modules of SolidsPack, employing the newest version of the file solidstandard.h, which is now incorporated into VnmrJ 3.2. These sequences stand alone in VnmrJ 3.2 so it is not necessary to load the SolidsPack software before running the BioSolids Pack experiments. All functions are now available through the individual sequence panels.

The sequences can be run in the BioPack environment, providing additional panels for multidimensional processing, including optional access to NMRPipe.

Table 1. Summary of multi-dimensional pulse sequences in BioSolids Pack. Polarization transfer pathways, most common experiments, and special features are noted.

A: ahXX



$H \xrightarrow{CP} C\{t_1\} \xrightarrow{MIX} C\{t_2\}$

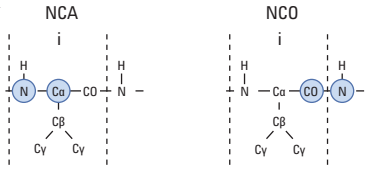
Features

Mixing options:
DARR, POST-C7, POST-C6, SPC5, RFDR, PARIS

Common experiments

2D: CC

B: ahYX



$H \xrightarrow{CP} N^{i}\{t_1\} \xrightarrow{CP} C_{\alpha}^{i}\{t_2\}$ $\quad H \longrightarrow N^{i}\{t_1\} \longrightarrow C'^{i}\{t_2\}$

Common experiments

2D: NCA, NCO

C: ahYXX

$H \xrightarrow{CP} N^{i}\{t_1\} \xrightarrow{CP} C_{\alpha}^{i}\{t_2\} \xrightarrow{MIX} C^{i}\{t_3\}$ $\quad H \xrightarrow{CP} N^{i}\{t_1\} \xrightarrow{CP} C'^{i-1}\{t_2\} \xrightarrow{MIX} C^{i-1}\{t_3\}$

Features

Mixing options:
DARR, POST-C7, SPC5

Band selective X (^{13}C) refocusing pulse

Constant time Y (^{15}N) evolution

Common experiments

3D: NCACX, NCOCX; 2D: (N)CACX, (N)COCX, N(CA)CX, N(CO)CX

D: ahXYX

$H \xrightarrow{CP} C_{\alpha}^{i}\{t_1\} \xrightarrow{CP} N^{i}\{t_2\} \xrightarrow{CP} C'^{i-1} \xrightarrow{MIX} C^{i-1}\{t_3\}$ $\quad H \xrightarrow{CP} C'^{i-1}\{t_1\} \xrightarrow{CP} N^{i}\{t_2\} \xrightarrow{CP} C_{\alpha}^{i} \xrightarrow{MIX} C^{i}\{t_3\}$

Features

Mixing option:
DARR

Band selective X (^{13}C) refocusing pulse

Constant time Y (^{15}N) evolution

Common experiments

3D: CANCO, CONCA, CAN(CO)CX, CON(CA)CX; 2D: CA(NCO)CX, CO(NCA)CX

3D Experiments with NCACX and NCOCX

The 3D experiments NCACX and NCOCX (Table 1C) are most commonly used to obtain resonance assignment data. Figure 1 shows the pulse-sequence diagram and typical strip plots for the NCOCX experiment for the immunoglobulin-binding domain B1 of streptococcal protein G (GB1). For this experiment, nitrogen-to-carbon correlation with either the alpha (NCA) or carbonyl (NCO) carbons establishes amino acid connectivity through selective double cross polarization (DCP). Carbon-carbon correlation to CX using a mixing sequence (in this case dipolar-assisted rotational resonance, (DARR)) identifies the amino acid side chain. The strip charts of Figure 1B represent each carbon-carbon 2D plane corresponding to an individual ^{15}N resonance.

Both NCACX and NCOCX are options of the pulse sequence **ahYXX**: **a** is a label for sequences of the package, **h** refers to the special role of protons for polarization only, **Y** refers to amide nitrogen, and **X** refers to either CA or CO. This experiment obtains its initial polarization for ^{15}N from protons and eventually detects ^{13}C magnetization. The first evolution period is for ^{15}N and the second for ^{13}C. The ^{13}C detection follows carbon-carbon mixing through DARR. A second pulse sequence, ahXYX (Table 1D), provides the additional CANCO, CONCA, CAN(CO)CX and CON(CA)CX experiments.

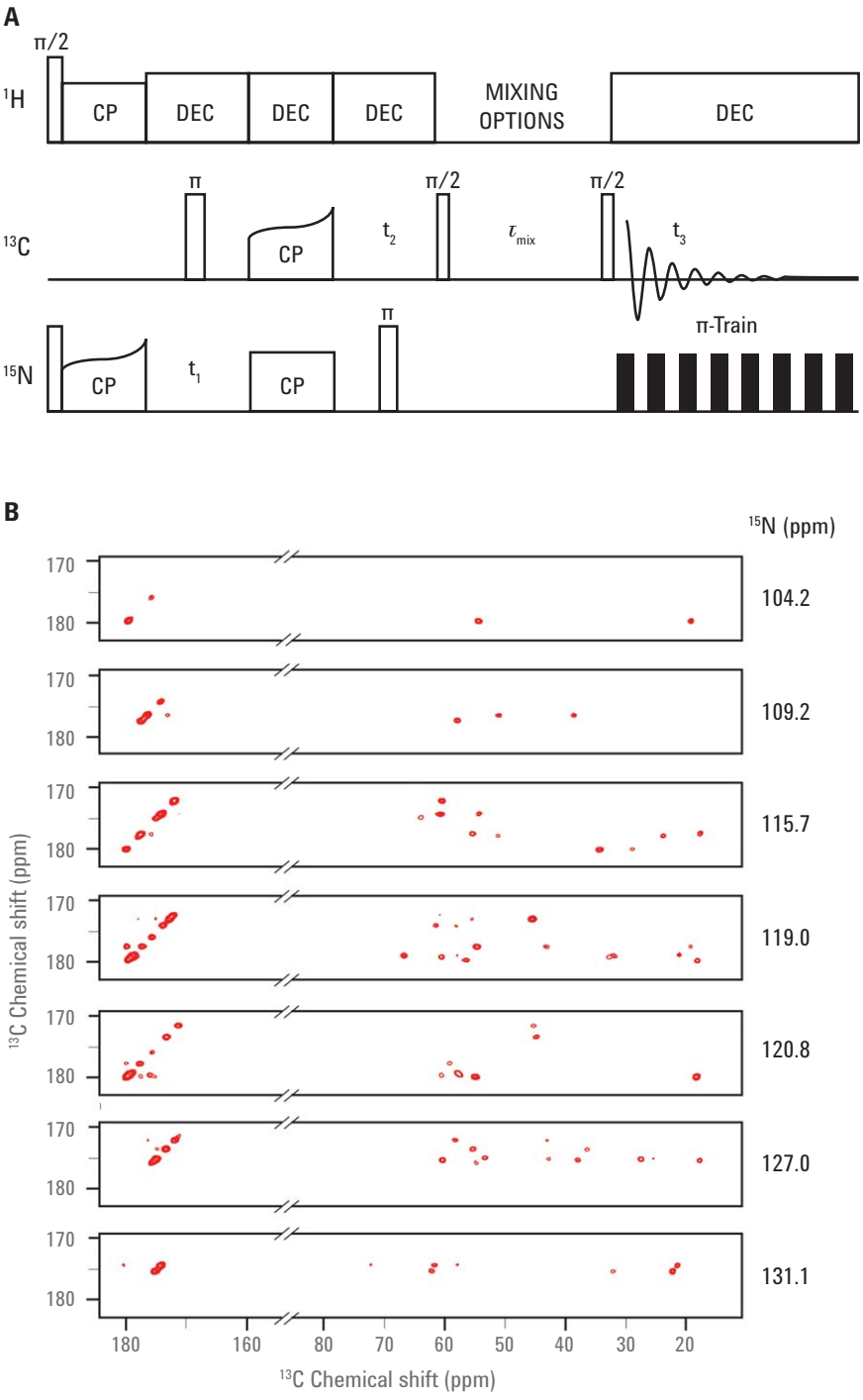


Figure 1. A pulse sequence diagram of the 3D NCOCX experiment (A) and strip plots obtained for the microcrystalline protein GB1 using a 600 MHz Agilent DD2 NMR system (B).

Multiple Sequences for Carbon-Carbon Mixing

The sequence ahYXX also provides a variety of optional carbon-carbon mixing sequences that may be chosen to best suit the individual application. Figure 2 shows ^{15}N-^{13}C 2D planes obtained with NCACX for three common mixing sequences: DARR, $SPC5_3$, and POST-C7. DARR is known to provide equal mixing among all carbon types. The $SPC5_3$ and POST-C7 mixing sequences are more band-selective, and for these experimental conditions they favor CA-CB and CA-CO transfer, respectively. The N(CA)CX 2D spectra in Figure 2 were obtained with constant time ^{15}N evolution and a soft pulse for selecting Cα. While DARR transferred polarization to both C′ and the side chains efficiently, POST-C7 and $SPC5_3$ are more band-selective mixing schemes. As shown in Figure 2, POST-C7 provided more efficient transfer to CO peaks than to Cβ peaks, and $SPC5_3$ gave more Cβ peaks than C′ peaks under the experimental conditions. The double quantum mixing as implemented in Figure 2 for the POST-C7 and $SPC5_3$ mixing sequences resulted in a change of sign for the CB and CO peaks, relative to CA.

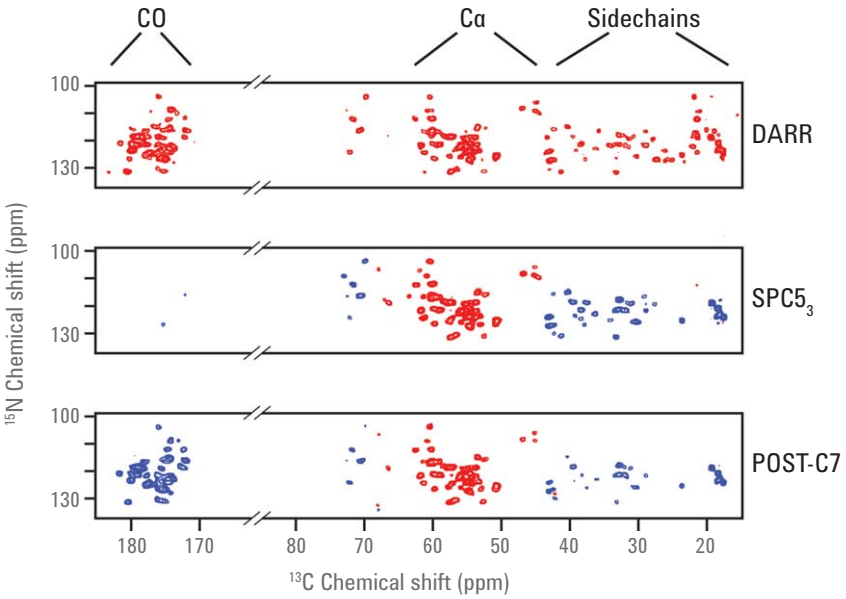


Figure 2. Different mixing schemes can be used to best suit the application as illustrated in GB1 N(CA)CX spectra at 11.7 T. *The GB1 spectra are courtesy of the Rienstra lab.*

Mixing sequences within a single pulse sequence are chosen via a flag parameter, with a separate parameter display for each in the experiment panels. $SPC5_3$ and POST-C7 can be chosen with double-quantum filtering, which is useful for both background suppression (e.g., to observe membrane protein signals in the presence of a large lipid background) and for calibration of excitation efficiency within the 3D pulse sequence.

Investigators favor a wide variety of mixing sequences for different applications and spinning rates. Adding a new mixing sequence to a pulse sequence such as ahYXX is straightforward in BioSolids Pack. Mixing sequences are modular and are created by the standard waveform software of SolidsPack, which is now part of the VnmrJ 3.2 pulse sequence language. A pulse sequence change of this type involves only a few lines of code and a small amount of panel editing.

Band-Selective and Constant-Time Evolution Periods

Band-selective shaped pulses are used during ^{13}C evolution to narrow the line widths via J decoupling and to remove unwanted correlations from the data. For example, Figure 3 shows the aliphatic region of an NCACX experiment obtained with GB1. The predominant source of correlations is the selective polarization of alpha carbons resulting from NCA polarization followed by DARR mixing. The circled correlations on the left of Figure 3 are due to alternate pathways resulting from direct transfer through the beta and other carbons, and some are folded from outside the spectral window. A band-selective ^{13}C refocusing pulse in the alpha region can remove these correlations, as shown in the plot on the right. The shaped pulse refocuses only the C-alpha polarization, thus removing unwanted correlations with other carbons. In this case the shaped pulse was an RSNOB pulse obtained by access to the Pbox shaped-pulse package in VnmrJ 3.2. In this application, the CA line width would also be narrowed up to ~90 Hz by removing the evolution due to CA-CO (55 Hz) and CA-CB (35 Hz) couplings; whether this benefit is fully realized depends upon the selected evolution time in t_2.

Band-selective refocusing is one of three options for evolution that can be chosen with a flag parameter. For any evolution period, the user can choose a standard delay with composite refocusing of heteronuclear J couplings. For Y evolution the user can also choose a constant-time option.

Shaped, soft pulses are calculated directly using a call to Pbox with a pulse-sequence statement, a practice that has become standard for VnmrJ solution-state spectroscopy sequences. Capability for soft pulse pulse-width measurements is provided by the solids calibration software.

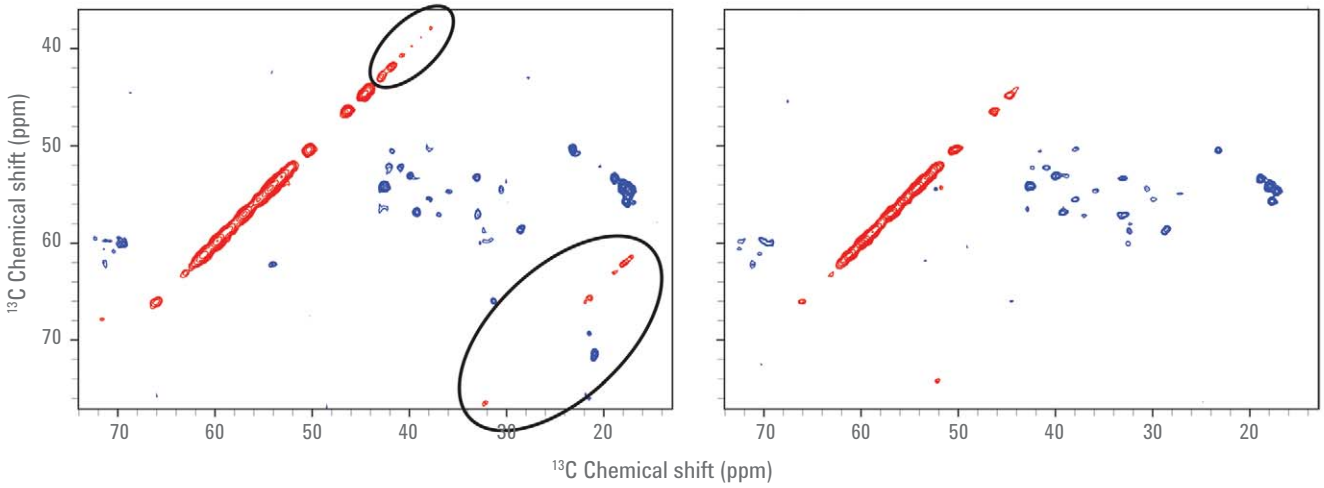


Figure 3. A pair of carbon-carbon 2D planes obtained using NCACX with DARR mixing for the protein GB1 at 11.7 T, with (right) and without (left) a C-alpha-selective refocusing pulse during ^{13}C evolution. *These spectra are courtesy of the Rienstra Lab.*

Pulse Sequence Calibrations

Figure 4 describes the 1D ahX sequence which is used to populate the probe file with calibrations for pulse widths, cross polarization, relaxation, and band-selective shaped pulses. As shown in Figure 4A, this single pulse sequence allows the choice of basic calibration experiments: standard cross polarization (CP), direct polarization, a CP with flip back for X pulse width, a Hahn echo for relaxation, and a soft echo to characterize refocusing pulse widths. A second sequence, ahYX (Table 1B), can be used for basic calibration of XY CP.

Figure 4B shows an example of a standard CP (top) and a CP with a soft refocusing pulse (bottom) as well as spectra obtained with the test sample N-acetylvaline (NAV). The soft-pulse option produces a selective spectrum (lower right), describing the bandwidth of the pulse and the efficiency of refocusing. In this case by using NAV, one can refocus > 94 % of the polarization relative to the CP spectrum above.

Calibrations within BioSolids Pack using ahX and ahYX are accomplished manually. However, an autocalibration package is available in SolidsPack.

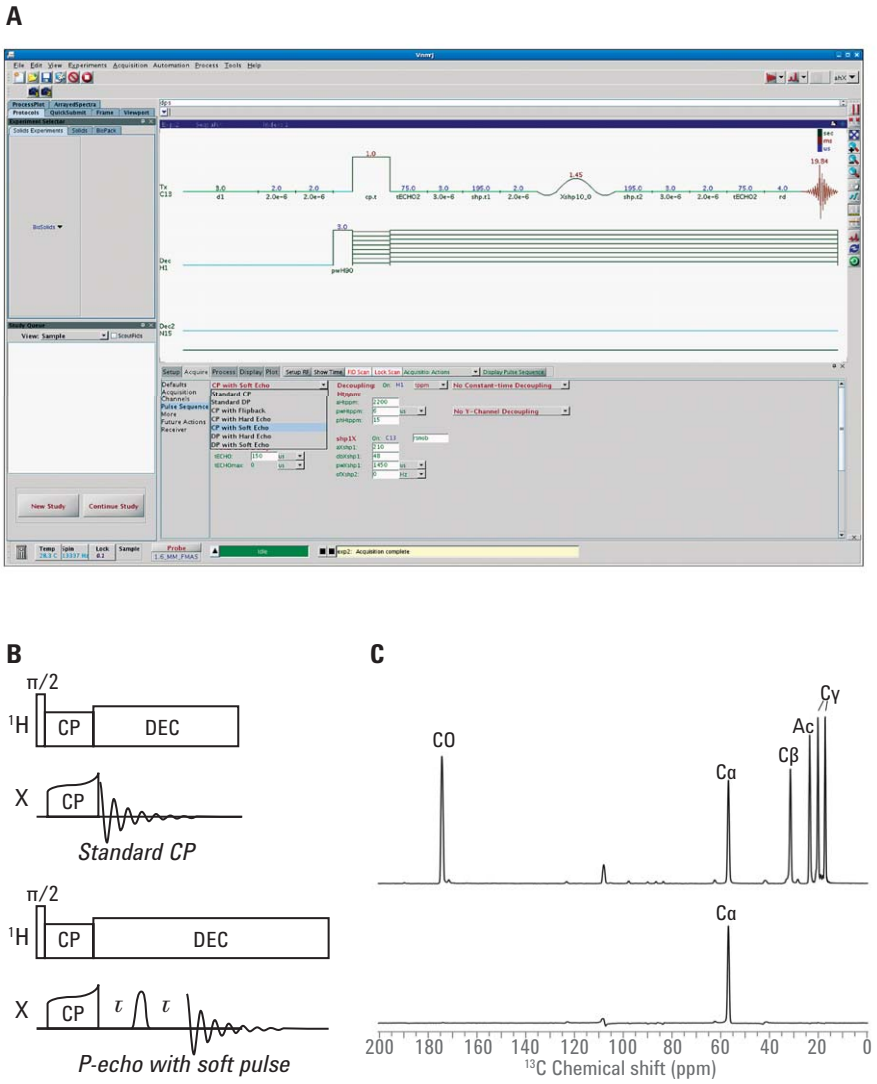


Figure 4. A VnmrJ 3.2 panel for the ahX sequence (A), showing how1D experiments are selected from the panel. Examples of a standard CP measurement and a soft-pulse measurement with resulting spectra of NAV (B).

Conclusions

This new BioSolids Pack is similar to BioPack and is intended to be a repository for Agilent users' BioSolids sequences. The sequences described here, using double CP and ^{13}C detection, have been provided by Professor Chad M. Rienstra's group at the University of Illinois, Urbana Champaign. These sequences, in their research versions, are already well-tested by the community and should be recognizable to many users. The package will grow, with additions for ^{1}H detection, plus J and TEDOR methods. Over time the BioSolids Pack will evolve to meet the critical need for solid-state biomolecular structural information.

Acknowledgement

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