NMR Spectroscopy

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Quadruple-Resonance Magic-Angle Spinning NMR Spectroscopy of Deuterated Solid Proteins**

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Abstract: *^{1}H-detected magic-angle spinning NMR experiments facilitate structural biology of solid proteins, which requires using deuterated proteins. However, often amide protons cannot be back-exchanged sufficiently, because of a possible lack of solvent exposure. For such systems, using ^{2}H excitation instead of ^{1}H excitation can be beneficial because of the larger abundance and shorter longitudinal relaxation time, T_1, of deuterium. A new structure determination approach, "quadruple-resonance NMR spectroscopy", is presented which relies on an efficient ^{2}H-excitation and ^{2}H-^{13}C cross-polarization (CP) step, combined with ^{1}H detection. We show that by using ^{2}H-excited experiments better sensitivity is possible on an SH3 sample recrystallized from 30% H_2O. For a membrane protein, the ABC transporter ArtMP in native lipid bilayers, different sets of signals can be observed from different initial polarization pathways, which can be evaluated further to extract structural properties.*

Solid-state NMR spectroscopy is becoming a routine tool for obtaining structures of solid biological macromolecules because of recent advancements in NMR methodology as well as hardware.[1] As a result, atomic resolution structures of polydisperse protein oligomers, protofibrils and membrane proteins have been obtained.[2] With the goal of increasing the sensitivity and information content of multi-dimensional NMR experiments, protein deuteration has been used to facilitate efficient amide proton detection in solid-state NMR spectroscopy. To make this work, it is mandatory that exchange of deuterons, which are present at the exchangeable sites after protein expression, with protons is efficient. A 30% presence of protons at exchangeable sites has been found to be appropriate for proteins fully deuterated at nonexchangeable sites (^{2}H/^{13}C/^{15}N, DCN) to achieve an optimal signal-to-noise ratio (S/N) at moderate MAS frequencies of 20–24 kHz, whereas 100% of the protons at the exchangeable sites can be used without sensitivity and resolution losses at 60 kHz MAS.[3] Under such conditions, experiments known from solution NMR spectroscopy can be applied to solid proteins. In most cases, however, pulse sequences employing cross-polarization (CP) units instead of INEPT schemes for transfer between different types of nuclei lead to better results.[4] Examples are the so-called out-and-back (HNC_α- or $HNCOC_\alpha$-type) or sidechain-to-backbone correlation ($C_\beta C_\alpha NH$-type) experiments.[3a,5] These approaches would work on standard DCN-labeled samples with an appropriate amount of protons at the amide sites. As a different approach, DCN-labeled samples with random residual protonation of carbon atoms to a certain degree may be applied to enable proton detection. However, this results in resolution losses because of signal splitting by isotope effects.[6] Alternatively, selective proton labeling of methyl groups may be applied.[7]

For many types of proteins that can only be studied in their native environments and that are difficult to unfold and refold to enable sufficient back-exchange of amide protons (e.g. membrane proteins), excitation of protons may not always result in maximum sensitivity. In such situations, it is of great interest to excite the abundant deuterium spins in preparation for multi-dimensional NMR experiments, for example, to polarize carbon atoms by deuterium and detect carbon atoms. We recently demonstrated that up to an amide protonation rate of 30%, deuterium excitation ($^{2}H\rightarrow^{13}C$ by CP, simultaneous proton and deuterium excitation, or triple-cross-polarization TCP, $^{1}H+^{2}H\rightarrow^{13}C$ CP) might result in a better S/N ratio relative to proton excitation ($^{1}H\rightarrow^{13}C$ CP) alone.[8]

When starting with deuterium magnetization, the $^{2}H\rightarrow^{13}C$ polarization transfer step needs to be efficient to ensure that the dCN experiment is recorded with high sensitivity. Again for ensuring good S/N, we combine deuterium excitation with proton detection in a dCNH ($^{2}H\rightarrow^{13}C\rightarrow^{15}N\rightarrow^{1}H$) manner. A similar transfer scheme is especially appropriate when deuteration is combined with a specific methyl protonation

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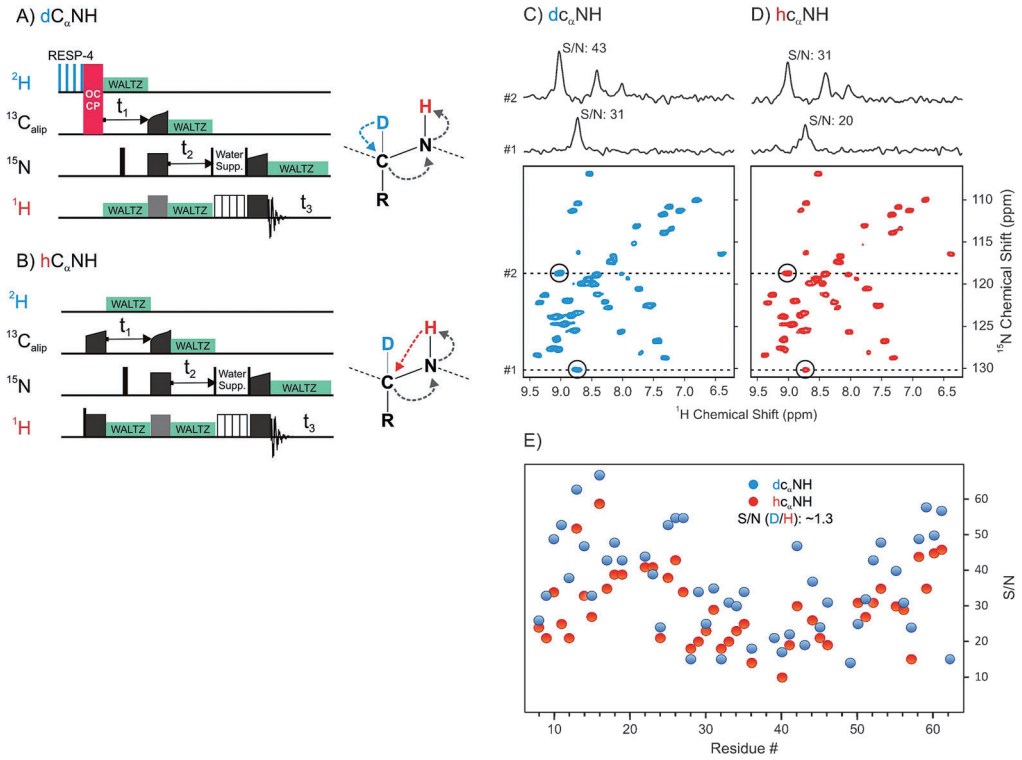

Figure 1. A,B) The pulse sequences used to record the dC$_\alpha$NH and hC$_\alpha$NH 2D and 3D MAS NMR experiments, respectively. RESP-4 refers to RESPIRATION 4 type of rotor-synchronized deuterium excitation pulses spanning four rotor periods C,D) The resulting HSQC-type proton-detected 2D d/hc$_\alpha$HN spectra of uniformly DCN-labeled SH3 with 30% exchangeable proton content. The spectra were plotted with the same contour levels to allow direct comparison at a contour cut-off of 10*sigma. Slices plotted with same noise level from two chemical shift values are added to the figure to judge the S/N differences. E) The comparison of the S/N extracted from 2D dc$_\alpha$NH and hc$_\alpha$NH experiments. The 2D spectra were recorded at 600 MHz, 20 kHz MAS, and 275 K using a quadruple resonance 3.2 mm Bruker MAS NMR probe. The 2D dc$_\alpha$NH and hc$_\alpha$NH spectra were recorded with 592 and 128 scans, respectively, with 128 transients for the indirect nitrogen dimensions (20 ms). Recycle delays of 0.5 and 3 s were used which led to total experimental times of 15.3 and 14.8 h for the 2D dc$_\alpha$NH and hc$_\alpha$NH spectra, respectively. 2D spectra were processed with 10 Hz Gaussian line-broadening in the ^{1}H dimensions and a 60° shifted squared sine bell function in the ^{15}N dimension.

scheme and methyl proton detection, leading to a dNCH experiment (^{2}H→^{15}N→^{13}C→^{1}H$_{metyhl}$). Overall, such a novel set of NMR experiments would lead to a new type of structure determination protocol for deuterated proteins lacking exchangeable protons.

Here, we take advantage of all four different nuclear spins present in perdeuterated proteins; protons, carbon atoms, nitrogen atoms, and deuterons (^{1}H, ^{13}C, ^{15}N, and ^{2}H), for recording 2D dc$_\alpha$NH and 3D dC$_\alpha$NH NMR spectra with better or comparable sensitivity than the equivalent hC$_\alpha$NH experiment (Figure 1 A,B), using a DCN-labeled SH3 domain sample. The method requires quadruple-resonance NMR hardware comprising four radiofrequency (RF) channels, and relies additionally on optimal control (OC) based excitation sequences capable of efficiently handling deuteron magnetization, such as the recently developed RESPIRATION ^{2}H excitation and optimal control ^{2}H-^{13}C ^{OC}CP methods.[9] Within the application shown here, proton detection schemes are employed to maximize sensitivity of the proposed quadruple-resonance NMR spectroscopy.

In proton-detected solid-state NMR spectroscopy, large sensitivity gains are achieved because of their high gyromagnetic ratio (γ). However, there are remarkably fewer protons than deuterons at sparse proton back-exchange ratios below or equal to 30%, the rate optimal for 20–24 kHz MAS.[3b] In this case, 70% or more C$_\alpha$ spins are not situated next to a proton, hence an important contribution for their excitation is missing in experiments employing H-C cross-polarization. Alternatively, deuterium excitation can be used efficiently for two reasons, despite its gyromagnetic ratio being seven times smaller than that of protons: 1) deuterium is more abundant in samples with low proton content, and even more importantly, 2) the longitudinal relaxation time (T_1) of deuterium nuclei (0.05–0.2 s) is much shorter than the T_1 of protons (2.8 s) in an undoped, 30% proton back-exchanged DCN-labeled SH3 sample. Considering that relaxation delays in experiments are optimal at $1.3 \times T_1$, the ratio of relaxation times yields a factor of 14 that favors deuterium excitation. In combination with the higher abundance of deuterium in such samples this results in larger overall sensitivity, as we have shown recently.[8,10] However, manipulating deuterium nuclei is more demanding with regards to excitation and polarization transfer, because of its large quadrupolar coupling (in the order of 160 kHz for the static case) and limited RF strengths available.[11] Nevertheless, our recently developed NMR toolbox for deuterium spectroscopy enable us to work with

deuterium and benefit from its large abundance and short relaxation times.

Figure 1 A,B shows the $dC_\alpha NH$ and $hC_\alpha NH$ pulse sequences used for proton-detected multidimensional MAS NMR spectroscopy, starting with deuterium and proton excitation, respectively. With these sequences, we recorded 2D $dc_\alpha NH$ and $hc_\alpha NH$ correlation spectra shown in Figure 1 C and D, respectively, at 600 MHz, 20 kHz MAS, and 275 K. The $dc_\alpha NH$ experiment uses ^2H RESPIRATION excitation and ^2H-^{13}C ^{OC}CP, which enabled the acquisition of high-sensitivity 1D and nD spectra with deuterium RF field strengths as low as 25 kHz and pulse delay of 0.5 seconds. The ability to use low (2.5 kHz) power ^1H decoupling allowed this short pulse delay without substantial sample heating. Sample heating because of fast repetition rates is not large enough to cause substantial chemical shift changes in the spectra of our SH3 sample, even for the temperature-sensitive residue T24 (see Figure S1 in the Supporting Information for the overlay of the 2D spectra). In general, more intense cross-peaks are obtained from the deuterium-excited experiment (Figure 1 E). Site-specific S/N values from identically processed 2D $dc_\alpha NH$ and $hc_\alpha NH$ experiments show an average S/N ratio of 1.3. With better instrumentation that allowed more RF input, the recycle delay for the deuteron experiments could be reduced to < 0.2 s, the actual $1.3 \times T_1$, which would lead to a more favorable S/N ratio for the deuterium-excited experiment compared to the proton-excited one. Both 2D spectra show average ^1H linewidths of 64 Hz and ^{15}N linewidths of 30 Hz (see Figure S2 in the Supporting Information for site-specific full-width-at-half maximum values, FWHM). A 3D $dC_\alpha NH$ spectrum obtained on the SH3 sample demonstrates the feasibility of performing deuterium-excited nD NMR spectroscopy with a sensitivity better than that of proton-excited NMR spectroscopy (the slices from the 3D $dC_\alpha NH$ spectrum are shown in Figure S3 in the Supporting Information).

The success of our approach depends strongly on the efficiency of the deuterium spectroscopy. Figure 2 shows the deuterium RF power dependence of the ^2H-^{13}C OC or of conventional CP experiments (Figure 2 A). Moreover, an initial ^2H-excitation by RESPIRATION and by a single 90° pulse (Figure 2 B) was tested. The integrals of the signals obtained from the experiments are shown on the lower plots (Figure 2 C and D) to quantify the results. RESPIRATION RF pulses and RESPIRATION ^{OC}CP methods render it possible to use low deuterium RF field strengths < 20–30 kHz (which is the instrumental limit of the quadruple resonance). At extreme RF strengths of 90 kHz (which is very demanding on many commercial NMR instruments) the conventional CP and excitation methods yield similar signal intensity to the OC and RESPIRATION methods. However, when the deuterium RF strength is decreased from 90 kHz to 50 kHz, the overall signal intensity decreases by 30–40 % for the conventional approach. Below 50 kHz deuterium RF strength, it is not possible to transfer polarization from deuterons to their attached carbon atoms using conventional CP (Figure 2 A). Here, we used shapes for ^{OC}CP that are optimized for deuterium RF strengths between 40–70 kHz, resulting in some signal reduction outside this RF range. This

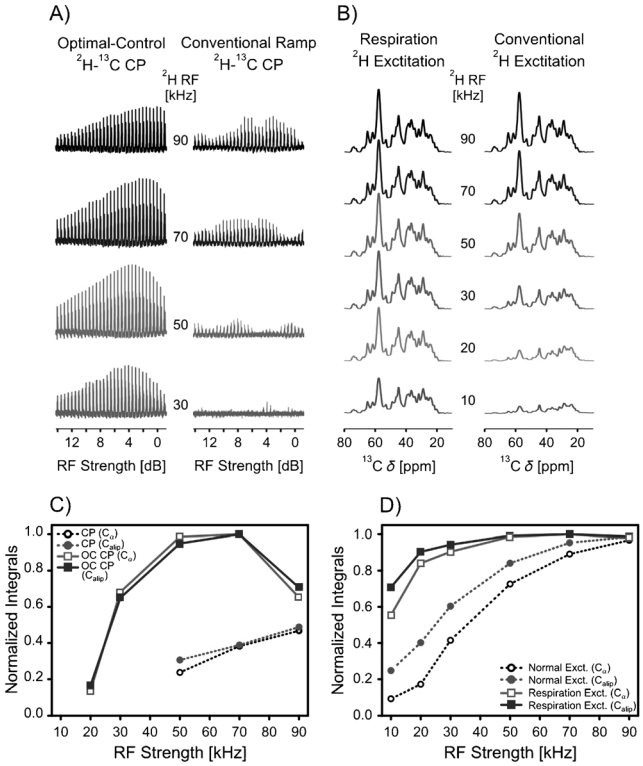

Figure 2. The representation of the effect of deuterium RF strength on deuterium-excitation and ^2H-^{13}C CP. A) For the ^2H-^{13}C CP, the effect of the RF strength is shown for the conventional-ramp and OC CP schemes. The ^2H CP RF strength is set to the given value, and the ^{13}C RF is arrayed over a wide RF range to obtain the largest possible ^{13}C CPMAS NMR signal. For these experiments the ^2H excitation RF was set to 90 kHz. B) For the deuterium excitation, the effect of RF strength is shown for the conventional and RESPIRATION 90°-excitation schemes with the given RF strengths, which uses the ^2H-^{13}C ^{OC}CP under the best matching condition obtained. For these experiments the ^2H RF for CP is set to 90 kHz. C,D) The integral values obtained from the spectra shown in (A,B) to aid in comparison. The spectra were recorded at 600 MHz, 20 kHz MAS, and 275 K. More of the experimental details are given in the Experimental Section in the Supporting Information.

effect could be eliminated with a shape optimization more suitable for lower deuterium RF strengths. A second important factor reducing sensitivity is the conventional initial deuterium excitation by a simple 90° pulse that becomes 50 % less efficient compared to RESPIRATION excitation. On the other hand, the deuterium excitation profile has very little signal reduction in the 20–90 kHz RF range when the RESPIRATION-4 deuterium-excitation is used.

In all NC_α, HC_α and NH planes from the 3D MAS NMR $dC_\alpha NH$ experiment recorded on DCN-labeled SH3, the resolution is similar to the ones recorded with an ultrafast 1.3 mm triple-resonance NMR probe. Similar 3D spectra were recorded for both proton- and deuterium-excitation versions. For the globular SH3 domain no differences were observed between those two spectra, because of the simple back-exchange procedure in this sample.

To further investigate the differences between ^1H and ^2H excitation in 3D $C_\alpha NH$ spectra, the experiments were

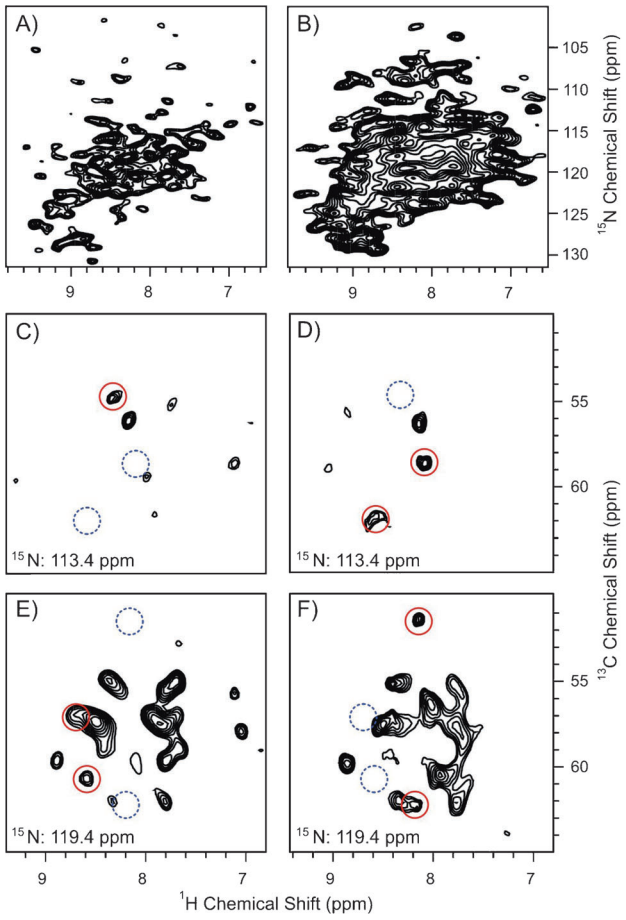

Figure 3. The 2D A) $dc_{\alpha}NH$ and B) $hc_{\alpha}NH$ MAS NMR spectra of the membrane protein ArtMP. The 2D ^{1}H-^{13}C planes from the 3D $dC_{\alpha}NH$ (C, E) and $hC_{\alpha}NH$ (D, F) experiments at different ^{15}N frequencies. The solid red circles indicate cross-peaks observed only in the deuterium- and proton-excited spectra, while the dotted blue circles indicate the corresponding location in the other spectra. The spectra were recorded at 600 MHz, 20 kHz MAS, and 275 K. The 2D planes were acquired in A) 15.5 and B) 15.8 h, while the 3D spectra took (C, E) 116 and (D, F) 96 h. 2D spectra (A, B) were processed with 60 and 30 Hz of net line-broadening in the ^{1}H and ^{15}N dimensions, respectively, and a 60° shifted sine-bell function in the ^{15}N dimension. 3D spectra (C, E) were processed with 50, 25, and 60 Hz of net line-broadening in the ^{13}C, ^{15}N, and ^{1}H dimensions, respectively, and a 80° shifted sine-bell function in the ^{13}C and ^{15}N dimensions.

performed on the fully DCN-labeled membrane protein complex ArtMP with 30% proton back-exchange. ArtMP is an ABC transporter from the thermophilic bacteria *Geobacillus stearothermophilus*. Figure 3 shows the first HN plane from $C_{\alpha}NH$ spectra recorded with A) ^{2}H and B) ^{1}H excitation. In contrast to what was observed for SH3, the excitation strategies yield very different results. In addition, the ^{2}H excitation experiment had less overall S/N and fewer signals than the ^{1}H excitation version. A comparison of the resolved cross-peaks from the 2D spectra shows that the S/N of the $dC_{\alpha}NH$ experiment is $0.5 \times$ S/N of the $hC_{\alpha}NH$. A large portion of this difference can be attributed to the shorter ^{1}H T_1 times in this more dynamic membrane protein sample relative to the microcrystalline SH3, and also partially to the use of protonated lipids for membrane reconstitution. Despite the sensitivity difference, we acquired the full 3D $dC_{\alpha}NH$ and $hC_{\alpha}NH$ spectra to investigate if regions of the protein were excited differently by the two methods.

Overall, the 3D $dC_{\alpha}NH$ and $hC_{\alpha}NH$ spectra showed 92 and 185 cross-peaks, respectively. Out of those, 28 and 94, respectively, could be correlated with residues in ArtP as assigned by solution NMR spectroscopy of the ArtP monomer. The peaks which do not correlate with the solution ArtP assignment are either from regions of ArtP that shift in the complex, or from ArtM itself (given that the residues identified in the 3D spectra span all of ArtP). Nearly half (49%) of the 3D $hC_{\alpha}NH$ cross-peaks and two thirds (70%) of the $dC_{\alpha}NH$ cross-peaks do not match to solution ArtP assignments, indicating that the $dC_{\alpha}NH$ experiment is more efficient at exciting residues likely to be from the membrane protein ArtM. Several possibilities exist to explain this observation. If the proton exchange at these sites is inefficient, local ^{1}H concentrations less than the desired 30% could be present. While still sufficient to enable ^{1}H detection, protonation at lower levels would yield the observed phenomenon both by lowering the efficiency of the initial ^{1}H-^{13}C cross polarization and lengthening the local ^{1}H T_1. Additionally, local dynamics in ArtM could interfere with the longer distance ^{1}H-^{13}C cross-polarization step, while not affecting the directly bonded ^{2}H-^{13}C and ^{15}N-^{1}H steps so dramatically. Accordingly, despite the lower overall signal to noise there are several examples of cross-peaks in the $dC_{\alpha}NH$ (Figure 3C,E) that do not appear in the $hC_{\alpha}NH$ (Figure 3D,F). Examples are cross-peaks at $\delta = 8.3/54.7$ ppm in Figure 3C and at $\delta = 8.6/60.8$ ppm in Figure 3E. This indicates clearly the usefulness of the deuterium-based multidimensional NMR spectroscopy to detect residues likely from the core of the membrane protein not seen in ^{1}H excited spectra. Conversely, some peaks appear in the $hC_{\alpha}NH$ that do not appear in the $dC_{\alpha}NH$, such at $\delta = 8.6/62$ ppm in Figure 3D and at $\delta = 8.05/51.5$ ppm in Figure 3F, presumably because of the better S/N of the proton-based experiment in regions of the complex more amenable to ^{1}H-based excitation.

In conclusion, we presented a new solid-state NMR approach, quadruple-resonance NMR spectroscopy, for experiments on perdeuterated proteins, and even more excitingly, for the ability to study systems (or specific parts of systems) which could not be back-exchanged sufficiently and as a result do not have enough protons at the amide sites for excitation. We showed that better sensitivity is possible on a fractionally back-exchanged (30%) SH3 protein by using deuterium-excited experiments (D→C→N or possibly D→N→C as well) compared to proton-excited ones (H→C→N). The results on the membrane protein ArtMP in native lipid bilayers emphasize that different sets of signals can be obtained from different initial polarization pathways, which can be evaluated further to extract structural properties from different sites of a protein (e.g. buried in the membrane versus more solvent exposed). Particularly, the parts outside the membrane (ArtP) likely showed more intense cross-peaks in the $hC_{\alpha}NH$ experiments, while the membrane buried parts (ArtM) may have benefited from the deuterium-excited version of the experiment, $dC_{\alpha}NH$. We anticipate that

quadruple-resonance NMR spectroscopy could be widely applied to a variety of interesting but difficult biological proteins, especially those which need to be studied in their native environments or which can hardly (or cannot at all) be back-exchanged, to obtain structural and dynamics information.

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