Protein NMR

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# Solid-State NMR of PEGylated Proteins

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***Abstract:*** *PEGylated proteins are widely used in biomedicine but, in spite of their importance, no atomic-level information is available since they are generally resistant to structural characterization approaches. PEGylated proteins are shown here to yield highly resolved solid-state NMR spectra, which allows assessment of the structural integrity of proteins when PEGylated for therapeutic or diagnostic use.*

Biological drugs (biologics)[1] are the fastest-growing category of approved therapeutics. Most biologics are proteins and, as such, they are the closest proxy of a "natural" help against disease.[2] However, proteins have poor pharmacokinetic and safety profiles. Polyethylene glycol (PEG) coating of biologics provides reduced renal clearance, increased stability to degradation, and a reduced immunogenic response (hence they are often called "stealth drugs").[3] PEGylation is even more important for nanocarriers and liposomes, the lifetimes of which are otherwise shortened by the activity of the reticuloendothelial system. Obviously, preservation of the three-dimensional structure and activity of the PEGylated form is mandatory for human use. While activity can be easily measured in vitro, structural characterization at atomic resolution of PEGylated proteins and protein-based nanocarriers is almost impossible. PEG coating prevents crystallization for X-ray analysis: currently only one crystal structure has been solved, that of the small protein PEG-plastocyanine (PEG-Pc, 11.5 kDa, 4R0O, 4.2 Å resolution). The protein carries a single PEG chain, and protein–protein crystal contacts can still take place owing to the rather large distance between the PEG moieties.[4] Also, PEGylation often pushes protein size beyond the practical limits of solution NMR because of the increase in hydrodynamic volume, which is increased by PEG more than it would be for an equal increase in protein mass.[5] Exceptions are PEG-Interferon$_{2\alpha}$[6] and PEG-Pc,[4] where the protein size remains sufficiently small after PEGylation to permit solution NMR studies. Solid-state NMR (ssNMR) is becoming a better alternative to study proteins[7] because it does not suffer from molecular-weight limitations (although the spectral complexity still increases with protein size), and high resolution spectra can be obtained for multimeric assemblies.[8–11] However, the best resolution is usually obtained for crystalline materials,[12,13] which are precisely what cannot be usually achieved for PEGylated proteins.

Herein, it is shown that highly resolved ssNMR spectra can be obtained for PEGylated proteins in the pelleted state, a densely packed non-crystalline state of a wet macromolecular sample, which can be obtained either by rehydration of freeze-dried material,[14–17] or by ultracentrifugation[18] when allowed by the density and molecular mass of the molecule.[19,20] Such high-quality ssNMR spectra are suitable for extensive resonance assignment and even conventional full structure determination. More importantly, the simple comparison of a standard two-dimensional ssNMR spectrum of the pelleted PEGylated protein with that of the crystalline state of the native protein—for which the X-ray structure is available—can reveal whether the three-dimensional structure is maintained in the PEGylated form. Since preparing a pelleted sample of a PEGylated protein turns out to be particularly simple and fast, and the collection of ssNMR spectra for the comparison is relatively rapid, the approach proposed here can be regarded as the fast-lane to assess at atomic detail whether or not the native conformation is preserved after PEGylation. Assignment of the resonances that experience perturbations, if any, can further push the application of this approach towards tracking where modifications occur in the 3D structure.

The approach is demonstrated on three clinically/preclinically relevant biologics: human $Cu^{II}$-$Zn^{II}$-Superoxide Dismutase (SOD), human H Ferritin (hHF), and *E.coli* L-asparaginase II (ANSII). SOD is an enzyme used to scavenge free radicals in radiation therapy and in severe inflammatory diseases.[21] The SOD used here is the popular "AS" C6A, C111S mutant, which is less prone to aggregation than the wild type. SOD is a symmetric homodimer ($C_2$) of 32 kDa that has been extensively characterized by NMR[22,23] and X-ray crystallography.[24] Ferritins have been proposed as biomineralization scaffolds, MRI contrast agents, and nanocarriers and drug delivery devices.[25] hHF is a homo-24-mer of about 504 kDa (ca. 21 kDa per subunit) that forms a cage of *O* symmetry (snub cube), as characterized by X-ray crystallography.[26] Its ssNMR spectra are here reported for the first time: it has the same architecture as the Bullfrog M ferritin,[27] with which sedimentation for ssNMR was first developed.[18] PEGylation increases the bloodstream circulation of ferritins and reduces specific uptake by cells.[28] ANSII from *E.coli* is in clinical use since 1967 against childhood acute lymphoblastic leukemia.[29] In its therapeutically active form, ANSII consists

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of a homotetrameric assembly of 144 kDa with $D_2$ symmetry.[30] For therapeutical applications, more recently, native ANSII has been substituted by the PEGylated form of ANSII, which exhibits longer-lasting activity and lower immunogenicity.[31]

Protein PEGylation was achieved by using a linear amine-reactive methyl-PEG reagent containing 24 ethyleneglycol units. PEGylation increases the molecular weight of dimeric SOD from 32 kDa to above 60 kDa, with all of the lysines and the N-terminal moiety largely functionalized (Figures S1, S2 in the Supporting Information). PEGylation of SOD hinders sedimentation, so the sample for ssNMR was prepared by wetting a freeze-dried sample. In hHF, each monomer binds 3–4 PEG chains, thereby increasing the size of the tetramer from 504 kDa up to 600–650 kDa (Figure S3) without changing its function (Figure S4–6). PEGylated hHF is still able to sediment easily. In ANSII, each monomer binds 4–5 PEG chains, thereby increasing the size of the tetramer from 144 kDa up to about 170 kDa (Figure S7). The ssNMR samples of native and PEGylated ANSII were both produced by freeze-drying and rehydration.

ssNMR spectra were collected for the three native proteins in the crystalline form and for the three PEGylated proteins in the pellet state (See Table S1 for detailed experimental conditions). From the analysis of the spectra (Figure 1 and S8) it clearly appears that line shape and resolution for the pelleted PEGylated proteins are very good, and comparable with those obtained from microcrystalline samples of the corresponding native proteins (Figure S8 and S9). Pelleted SOD and hHF in the native and PEGylated forms show spectra of equally good quality (Figure S10).

In all three cases, the spectra of the pelleted PEGylated protein and the crystalline native protein are largely superimposable, thus demonstrating for the first time a negligible effect of PEG coating on protein structure and assembly. In particular, preservation of chemical-shift patterns of well-resolved isoleucine residues indicates an intact hydrophobic core (Figure 1 and Figure S8). Figure 2 shows a superimposition of the spectra for pelleted PEGylated (blue) and crystalline native (red) SOD, thus demonstrating the remarkable similarity and the outstanding resolution achieved. The availability of the assignment of crystalline SOD also allows evaluation of the efficacy and sequence specificity of PEGylation by monitoring the chemical shift of the protein residues at the reaction sites (Figure 2B and Table S2). The residues experiencing non-negligible chemical-shift perturbations are mapped onto the SOD structure in Figure 2C. Chemical-shift perturbations mainly occur for surface residues, as expected. Some perturbations are observed for residues in close proximity to, or facing, the PEGylated lysines, while some are not obviously related to it.

As an illustration of the suitability of $^{13}C$-$^{13}C$ solid-state NMR for this kind of applications, Figure S11 shows the dramatic changes that are observed in the 2D $^{13}C$-$^{13}C$ correlation spectra of SOD upon chemical denaturation of the enzyme.[32]

The simplicity of the method should make it attractive for industrial purposes: biologics are expensive drugs, in terms both of design and of manufacturing, and significant savings could be obtained if ssNMR could be used to monitor optimization of the PEGylation procedure.

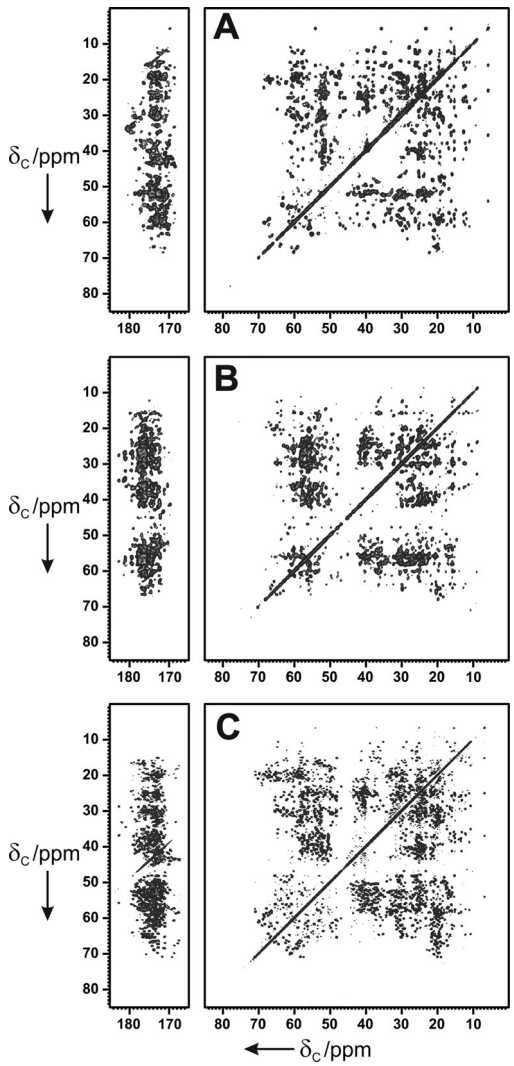


***Figure 1.*** 2D $^{13}C$-$^{13}C$ correlation spectra of PEGylated SOD (A), PEGylated hHF (B), and PEGylated ANSII (C). Conditions: A) 16.4 T, 14 kHz MAS, 3.2 mm rotor; B) 16.4 T, 11.5 kHz MAS, 4 mm rotor; C) 20 T, 14 kHz MAS, 3.2 mm rotor (Table S1).

The very good quality of the spectra and the high filling factor intrinsic to protein pellets[33] also make it possible to acquire 3D spectra for assignment (Figure S12). Since almost all carbon atoms can be observed with simple uniform $^{13}C/^{15}N$ labeling, and assignment does not need to rely upon extensive mutagenesis and/or selective labeling, as needed, for example, for methyl-TROSY,[34] for example, ssNMR is expected to lift the limitations for the study of PEGylated biologics.

It is important to remark that the outcome of the analysis does not strictly depend on the type of experiment used:

a) Historically, $^{13}C$-$^{13}C$ correlation spectra,[36,37] which are sensitive to changes occurring in the secondary structure, mostly through the Cα–Cβ region, and allow residue-type fingerprinting,[38–40] were used for spectral comparison.
b) With the introduction of robust NC recoupling schemes,[41,42] such comparisons are made even easier.

  

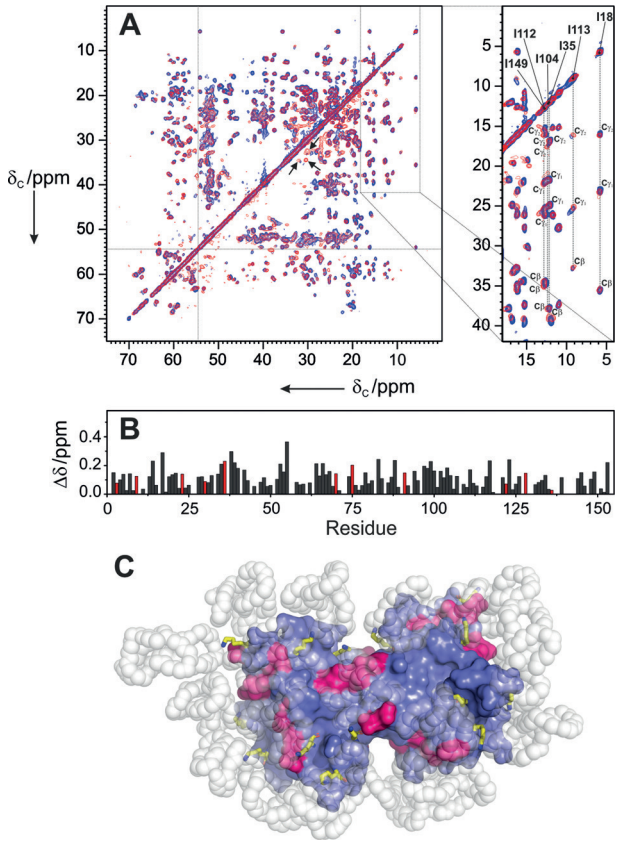


***Figure 2.*** A) Comparison of the aliphatic portion of the $^{13}C$-$^{13}C$ correlation spectra for crystalline native SOD (red) and pelleted PEGylated SOD (blue). Off-diagonal regions separated by dashed gray lines in the left panel are plotted with lowered intensities for the PEGylated form to ease comparison between the spectra of the two forms. Resonances marked by arrows belong to glutamate residues neighboring reacted lysine residues. The right panel shows an enlargement of the isoleucine Cδ1 resonances and correlations. B) chemical-shift perturbation between the two species ($\Delta\delta = \frac{1}{2}\sqrt{\Delta\delta^2_{C\alpha} + \Delta\delta^2_{C\beta}}$),[35] where lysine residues are highlighted in red. C) Structure of the PEGylated SOD homodimer (in blue, from the crystal structure) showing the lysine residues as yellow stick models, and the PEG chains (modelled as random chains) as white transparent spheres. The regions with non-negligible chemical shift perturbation of the side chains are shown in magenta.

Indeed, the NC correlation spectrum of ANSII shows remarkably resolved resonances (Figure S14).

c) More recently, $^{1}H$-detection-based ssNMR methods have been proposed,[43–45] and it is possible to foresee that comparison of $^{1}H$-$^{13}C$ or $^{1}H$-$^{15}N$ experiments could become the standard, yielding more sensitive detection on even lower amounts of sample.

Research on biologics is progressing towards the targeting of protein–protein interactions, which requires an even tighter control over the PEGylation processes.[46,47] The possibility of structurally characterizing PEGylated proteins represents a powerful tool for pushing the envelope of structural biology applications to biologics.


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