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NMR Spectroscopy Can Characterize Proteins Encapsulated in a Sol-Gel Matrix

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Proteins encapsulated within the pores of a sol−gel (SG) matrix retain structural integrity and functionality and are accessible to small molecules diffused into the gel, all of which enable applications in biosensors and biotechnology.[1−11] In addition, SG confinement can be used to control both inter- and intramolecular reactions[12−14] and to constrain conformational interconversion, both of which allow novel studies of kinetic intermediates in ligand-binding and protein folding.[15−17] The many applications of SG encapsulation are hindered, however, by the limited means of probing possible structural consequences of encapsulation. Optical, Raman, and CD techniques[5,12−16,18,19] are useful but are not as atomically informative as is NMR spectroscopy. Although NMR techniques have been used extensively to study the SG matrix, and solvents or gases that fill the SG "cells",[20] we are aware of only one previous solution NMR examination of small molecule probes or solutes encapsulated in a SG.[21] We here present the first demonstration that it is possible to acquire high-resolution, solution NMR measurements of SG-encapsulated proteins. This demonstration opens the way to detailed NMR-structural studies of all the above classes of encapsulated proteins, for example, encapsulated enzymes and kinetic intermediates.

With the aim of determining the breadth of an NMR approach, we have encapsulated three paramagnetic proteins with different overall charges: the highly acidic human Fe^{3+} cytochrome b_5 (cyt b_5),[22] molecular weight of ∼10 kDa and pI of ∼4.6; the highly basic horse heart cytochrome *c* (cyt *c*), ∼12 kDa and pI = 9.45;[23] the nearly neutral, sperm whale cyanomet-myoglobin, ∼16 kDa and pI of ∼7.5. Paramagnetic proteins were used because of the ease in assigning contact-shifted peaks.

NMR samples of proteins encapsulated in deuterated SGs were prepared using a variation of the procedure of Ellerby et al.[5] by mixing d_{12}-tetramethyl orthosilicate (TMOS) (Aldrich) with 0.1 M DCl until homogeneous, then adding a D_2O/buffered stock solution of protein; conditions for preparing SGs with the three individual proteins are given in the legends to Figures 1 and 2. SG samples for NMR measurements must be of higher quality than those for optical measurements. For the latter, one needs only a clear volume region for light transmission; cracks and cloudy regions elsewhere can be ignored. However, any inhomogeneities in a gel can contribute to field inhomogeneity and thus NMR line-broadening. The difficulty in preparing SGs for NMR is compounded because gelation times for d_{12}-TMOS SGs are significantly shorter than for the protonated SGs. This forced us to prepare the SGs at 4 °C, where a semisolid SG forms within minutes (depending on buffer conditions). SGs continue to ripen after initial gelation;[1] the NMR spectra obtained from a SG sample remained invariant over a period of ∼1 h to ∼1−2 weeks after the initiation of gelation. SG aged for a longer time did not yield good spectra.

Figures 1 and 2 present proton NMR spectra of SG-encapsulated cyt b_5, and Mb, along with the corresponding spectra of an aqueous solution; analogous spectra for cyt *c* are available in Figure S1.

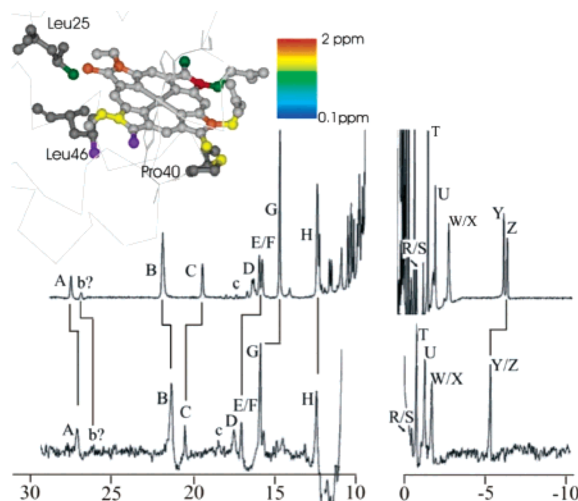

Figure 1. 1D comparison of sol−gel and aqueous spectra of cyt b_5 (conditions: 100 mM potassium phosphate at pH 6.25). All protein concentrations are ∼1 mM in 99.8% deuterium oxide and pH values were read straight from the pH meter without correction for the deuterium shift. All NMR spectra were collected at 35 °C with a 600 MHz Varian INOVA600 spectrometer equipped with a 5 mm triple-resonance gradient probe. The inset shows peak shifts upon encapsulation. The heme, along with any amino acids with peaks that clearly appear in the SG spectra, are shown in gray. Peak shifts are colored appropriately. (PDB ID: 1CYO).

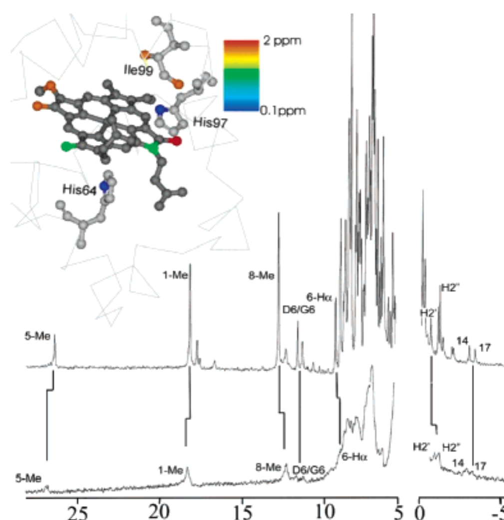

Figure 2. 1D comparison of sol−gel and aqueous spectra of Mb (conditions: 50 mM potassium phosphate + 50 mM KCN at pH 7.0). The inset shows peak shifts upon encapsulation. The heme, along with amino acids with assigned peaks in the SG spectra, are shown in gray. Peak shifts are colored appropriately. (PDB ID: 1YMB).

Despite the use of d_{12}- TMOS and D_2O buffers and presat or WATERGATE solvent suppression methods, the region between ∼5 to 0 ppm in the SG spectra is obscured by the large ^1H signals

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from H_2O and the primary methyls of TMOS. However, the contact-shifted peaks for both cyt b_5 and Mb that fall outside this window are well resolved. In contrast, the spectrum of cyt *c* shows only 3−4 very broad, shifted peaks. Figures 1 and 2 include literature assignments of the contact-shifted resonances of the heme and nearby amino acids for the solution proteins, along with proposed correspondences for the proteins in SG.[24−26] These findings parallel earlier ones that show negative and neutral (or nearly neutral) solutes are free to tumble when encapsulated, while cationic solutes interact strongly with the anionic silicate walls.[21,27−32]

The resolved peaks of SG-encapsulated cyt b_5 and Mb have nearly 2-fold greater linewidths than those of the solution spectra (∼1.6× for cyt b_5; ∼1.9× for Mb). There are two likely sources of this increase: magnetic susceptibility dispersion introduced by inhomogeneities in the SG; increases in rotational correlation times (τ_c) caused by encapsulation. The fractional changes in τ_c upon encapsulation were determined through measurements of T_1 for multiple contact-shifted peaks of cyt b_5 and Mb by use of the inversion−recovery technique. The average T_1 for the sampled contact-shifted peaks decreases roughly 2-fold upon encapsulation, from $T_1 = 1.6/22$ ms for cyt b_5/Mb in aqueous buffer, to $T_1 = 0.7/11$ ms in SG. Simple theories of paramagnetic relaxation give $1/T_1$ proportional to τ_c.[33] The SG encapsulation thus causes a corresponding 2-fold increase in τ_c, which accounts for the entire broadening. This increase in τ_c for proteins of 10−16 kDa is essentially the same as that observed for encapsulated anionic and neutral small molecules, ∼0.1 kD. The absence of a significant effect of molecular weight on molecular rotation suggests that the SG cage does not "straight-jacket" an encapsulated protein. Rather the increase in τ_c suggests that the solvent within a cage acts as if it had a roughly 2-fold greater viscosity than that of the bulk.

The poorly resolved spectrum of the cationic cyt *c* (Figure S1), however, tells a different story of strong electrostatic interactions with the anionic silicate walls that strongly hinder rotation. Indeed, with only a few peaks and such large linewidths in the SG spectrum, we were unable to measure T_1 for the encapsulated protein. These observations parallel those upon encapsulation of small cationic molecules, where the electrostatic attraction between probe and the negatively charged SG walls causes a large population of the probes to become effectively immobilized and/or to undergo transient immobilization.[21,27−31]

In addition to broadened lines, the contact-shifted peaks of the encapsulated proteins show small, but significant, shifts of as much as ∼1.9 ppm (Figure 2).[34] As shown in Figure 2, shifted ^{1}H-peaks are not associated with the protein surface and hence likely reflect small structural changes upon encapsulation. How such perturbations arise in an essentially freely rotating protein is far from clear. Prior studies note the presence of methanol produced upon gelation;[35] we find that the NMR spectrum of Mb in solution is unperturbed by 10% methanol. Preliminary 2D ^{1}H-NOESY and ^{13}C-HSQC spectra with unlabeled cyt b_5 and Mb showed a few cross-peaks; these techniques should be applicable with isotopically labeled proteins.

In summary, we have shown that it is possible to obtain well-resolved solution NMR spectra of SG-encapsulated proteins and suggest that extensive structural studies will be possible with labeled proteins. Encapsulated anionic and neutral proteins undergo essentially free rotation and show minor conformational perturbations as revealed by shifts of contact-shifted peaks associated with the heme and nearby amino acids. Henceforth, it will be possible to use NMR methods to directly probe the structural integrity of proteins used in applications of SG encapsulation techniques. Perhaps most promising, the combination of SG-encapsulation and NMR spectroscopy can provide detailed structural data on intermediates formed by ligand photolysis and give snapshots of conformational changes during ligand rebinding.[14,16]

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Supporting Information Available: A complete table of shifts and assignments for the peaks labeled in Figures 1 and 2, plus the cyt *c* spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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