Location of Fluorotryptophan Sequestered in an Amphiphilic Nanoparticle by Rotational-Echo Double-Resonance NMR

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ABSTRACT Rotational-echo double-resonance (REDOR) ^{13}C NMR spectra (with ^{19}F dephasing) have been obtained of 6-fluorotryptophan complexed by a polymeric amphiphilic nanosphere consisting of a polystyrene core covalently attached to a poly(acrylic acid)-polyacrylamide shell. The REDOR spectra show that aromatic carbons from the polystyrene core and oxygenated carbons in the poly(acrylic acid)-polyacrylamide shell are both proximate to the ^{19}F of 6-fluorotryptophan. Molecular modeling restrained by distances inferred from the REDOR spectra suggests that all of the 6-fluorotryptophans are in the shell but within 10 Å of the core-shell interface.

Water-soluble, amphiphilic polymer nanospheres can be synthesized (Thurmond et al., 1996) with a hydrophobic polystyrene core and a cross-linked poly(acrylic acid)-polyacrylamide shell (Huang et al., 1997). Each core polymer chain is covalently attached to the cross-linked weblike shell (Fig. 1). The hydrophilic functionality of the surface can be modified to attract either cations or anions. The entire unit is called a shell cross-linked knedel (SCK) ("knedel" is a Polish word for a dumpling with a fruit or meat center; Latterman, 1997). Dendrimers, cationic liposomes, and surface-active particles like SCKs can all complex DNA (van der Woude et al., 1995; Kukowskalatallo et al., 1996). SCKs are attractive to work with because of their mechanical and chemical stability, and because their surfaces are conveniently modified (Huang et al., 1997). SCK-DNA complexes with covalently attached recognition factors are of some practical interest because of their potential as transfection vehicles in gene therapy (Behr, 1994).

SCKs also have applicability for targeted drug delivery and sequestration of metabolites. The SCK structure is similar to a polymer micelle, except that the peripheral hydrophilic layer is a cross-linked matrix. It is well known that small hydrophobic molecules can move from aqueous solution, through the corona of polymer micelles, and into the core domain (Arca et al., 1995). However, the effects of the cross-linked matrix layer of an SCK on the transport of small molecules from water solution to the hydrophobic core of the nanoparticle are uncertain. Characterization of the location of the drug or metabolite within either the core or shell of the SCK is difficult by conventional biophysical methods. In this communication we demonstrate the use of $^{13}C\{^{19}F\}$ rotational-echo double resonance (REDOR, Gullion and Schaefer, 1989a, b) to locate amphiphilic 6-fluorotryptophan that had been sequestered from a water-tetrahydrofuran solution by an SCK.

Received for publication 11 May 1998 and in final form 28 July 1998.

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0006-3495/98/11/2574/03 $2.00

The SCKs were prepared from polystyrene$_{130}$-*b*-poly(acrylic acid)$_{120}$ diblock copolymers having 130 polystyrene repeat units and 120 poly(acrylic acid) repeat units. The poly(acrylic acid) chains located within the shell of the polymer micelles were cross-linked with 2,2′-(ethylenedioxy)bis(ethylamine), resulting in the conversion of >90% of the acrylic acid functional groups to amide linkages. The cores of the SCKs are rigid at room temperature because of the high glass-transition temperature of polystyrene (T_g = 105°C). An SCK complex was formed by adding 6-fluorotryptophan (10 wt %) to a solution of SCKs in a mixture of water and tetrahydrofuran (20% tetrahydrofuran/H_2O). Water swells the poly(acrylic acid)-polyacrylamide shell and tetrahydrofuran solvates the polystyrene core so that the 6-fluorotryptophan was complexed under conditions that potentially allowed for loading throughout the SCK. After 12 h, the tetrahydrofuran was removed by evaporation under vacuum. The remaining aqueous solution of 6-fluorotryptophan-SCK complex was frozen rapidly and the water was removed by lyophilization. The resulting powder was packed into a 7.5-mm outside-diameter zirconia rotor for examination by solid-state NMR. REDOR experiments were performed with 5-kHz magic angle spinning, a single 10-μs refocusing ^{13}C π pulse, and xy8 phase cycling of the 10-μs, rotor-synchronized ^{19}F π pulses (Wooley et al., 1997).

The REDOR full-echo spectrum (Fig. 2, *bottom*) shows resolved peaks associated with each of the components of the SCK complex. The REDOR difference spectrum (Fig. 2, *top*) has substantial contributions from carbons that are in the polystyrene core as well as from carbons in the poly(acrylic acid)-polyacrylamide shell. Qualitatively, this result indicates that some of the fluorotryptophan must be near the core-shell interface (Tong and Schaefer, 1997). The radius of the SCK is 14 nm (Fig. 1), which means that the core has a radius of 11 nm (calculated from the SCK radius, total composition, and component densities). If 6-fluorotryptophan were predominantly near the outer surface of the SCK ~3 nm from the nearest polystyrene in the core, no REDOR dephasing would be observed for the aromatic-carbon peaks. The range of $^{13}C\{^{19}F\}$ REDOR is ~12 Å (Holl et al., 1992).

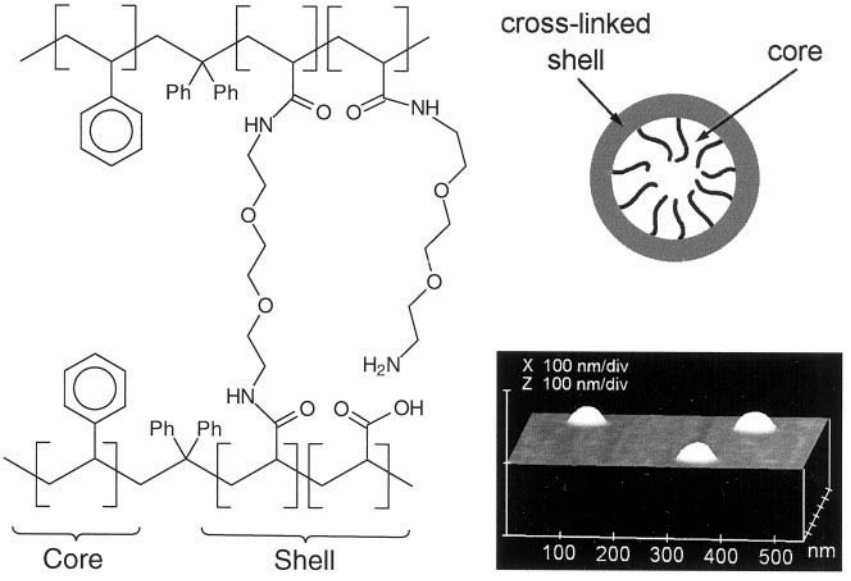

FIGURE 1 (*Left*) Chemical structure of an SCK having a polystyrene core (130 repeat units) and a poly(acrylic acid)-polyacrylamide shell (120 repeat units). The shell is stabilized by reaction with 2,2′-(ethylenedioxy)bis(ethylamine), an ethylene oxide cross-linker. For the SCK used in this study, >90% of the acrylic acid groups were converted to acrylamidines. (*Bottom right*) Atomic force microscopy image (Huang et al., 1997) of three 28-nm diameter SCKs (whose composition matches that of the structure on the left) adsorbed on mica under air. The *x* and *z* axes of the image are each 100 nm per division. (*Top right*) Schematic drawing of an SCK having an outside diameter of 28 nm and a core diameter of 22 nm. All of the carbons of the shell are within ~30 Å of the surface of the core. The curved lines inside the core represent individual polystyrene chains.

Simple modeling was done in an effort to make the positioning of the 6-fluorotryptophan within the SCK more quantitative. If all 1600 fluorotryptophans within each knedel (based on composition) are assumed to be located on the surface of a sphere centered at the center of the SCK, and with a radius of 11.5 nm (Fig. 3, *top*), then the average F-F spacing is ~8 Å. This packing is too tight for the observed ^{19}F-^{19}F dipolar coupling of only 100 Hz (data not shown), even though this arrangement of fluorines accounts reasonably well for the aromatic and oxygenated-carbon REDOR dephasing ($\Delta S/S_0$) as a function of the total number of rotor cycles of dipolar evolution (Fig. 4, *solid lines*). Alternatively, a distribution that has one-fourth of the fluorotryptophans on the polystyrene core surface, one-half on a sphere of radius 11.5 nm, and one-fourth on a sphere of radius 12 nm (Fig. 3, *bottom*), increases the F-F distance to >12 Å, and still accounts reasonably well for the REDOR dephasing for both shell and core (Fig. 4, *dotted lines*). Any sort of broader distribution places more fluorotryptophans farther away from the core, fails to account for the aromatic-carbon dephasing, and predicts too much oxygenated-carbon dephasing. We conclude that the tryptophans are all in the shell but within 10 Å of the SCK core-shell interface.

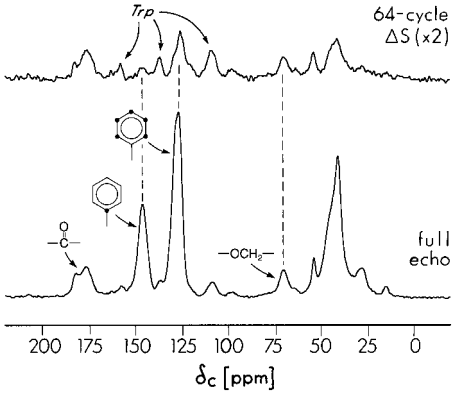

FIGURE 2 50.3-MHz ^{13}C REDOR NMR spectra (with ^{19}F dephasing) of 6-fluorotryptophan sequestered from solution by a 28-nm diameter SCK. The full-echo spectrum (S_0) after 64 rotor cycles with magic-angle spinning at 5 kHz is at the bottom of the figure and the REDOR difference (ΔS, x2) is at the top. The REDOR difference is $S_0 - S$, where S_0 is the full-echo spectrum obtained without dephasing pulses, and S is the reduced-echo spectrum obtained with dephasing pulses. Arrows mark the 146-ppm nonprotonated and 127-ppm protonated aromatic-carbon peaks of the polystyrene core, and the 70-ppm oxygenated-carbon peak of the cross-linker in the shell. All three of these peaks have REDOR differences indicating that at least some of the 6-fluorotryptophan is near the core-shell interface. The 145-ppm nonprotonated aromatic-carbon peak of the core is in a region of the spectrum that has no contribution from fluorotryptophan aromatic carbons; the 127-ppm REDOR difference peak has minor contributions (high and low-field shoulders) from fluorotryptophan. Resolved peaks from 6-fluorotryptophan are at 109 ppm (two carbons), 137 ppm, and 159 ppm. A minor methyl-carbon peak at 15 ppm is due to residual carbodiimide (used in the amide-bond cross-linking), which was not completely removed by the dialysis that preceded complex formation.

Important parameters that affect the sequestration of drugs and metabolites by SCKs include the compositions of the core, shell, and absorbed species, the cross-link density of the shell, the concentration of unreacted amines within and on the shell, and the composition of the sequestering solution. These variables are being examined in REDOR experiments now in progress that involve the use of stable-isotope labels in both core and shell, as well as in the absorbed species.

This work was supported by NSF Grants DMR-9458025 (to K.L.W.) and MCB-9404860 (to J.S.) and by a grant from the Monsanto Company.

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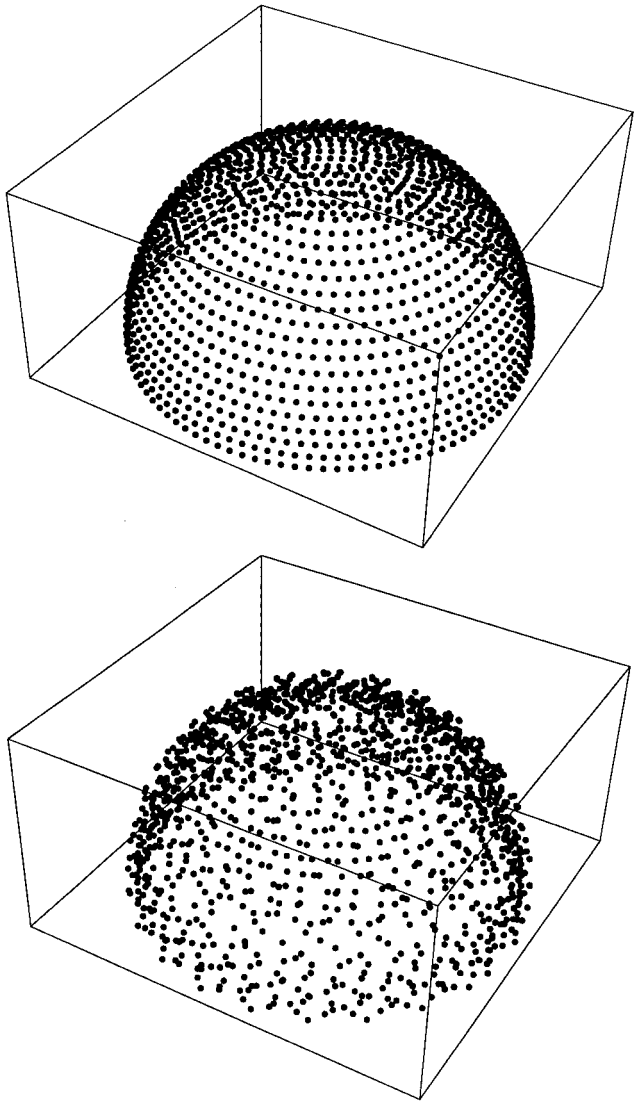

FIGURE 3 (*Top*) Distribution of fluorines (*solid circles*) of 1600 6-fluorotryptophans placed uniformly on a sphere of radius 11.5 nm. Only a hemisphere is shown for clarity. (*Bottom*) Distribution of fluorines of 1600 6-fluorotryptophans placed on three concentric spheres with the same center. One-fourth of the 6-fluorotryptophans are distributed uniformly on a sphere of radius 11.0 nm, one-half on a sphere of radius 11.5 nm, and one-fourth on a sphere of radius 12.0 nm. Only a hemisphere is shown for clarity.

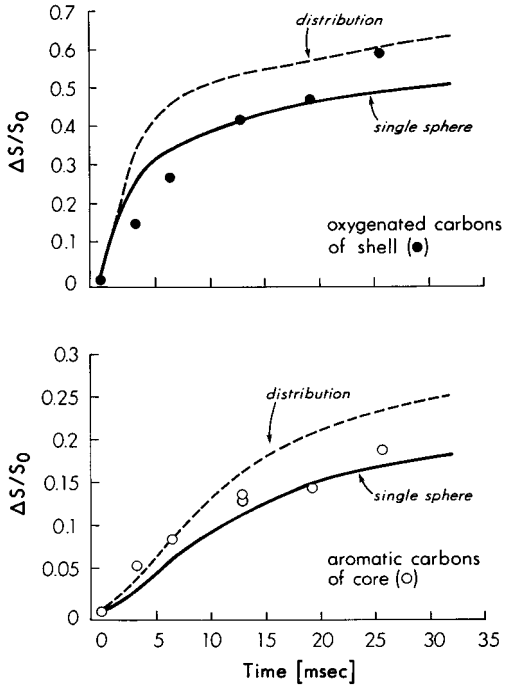

FIGURE 4 $^{13}C\{^{19}F\}$ REDOR dephasing ($\Delta S/S_0$) as a function of the dipolar evolution time for oxygenated carbons of the ethylene oxide cross-linker within the shell (*top, solid circles*) and protonated aromatic carbons of the core (*bottom, open circles*) of the SCK-6-fluorotryptophan complex of Fig. 2. The dotted and solid lines show the predicted dephasing assuming the arrangements of fluorines of Fig. 3. The calculations were done using the multi-spin methodology of Goetz and Schaefer (1997), under the assumption that all the effects of the 100-Hz ^{19}F-^{19}F coupling were suppressed by magic-angle spinning.

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