REDOR Determination of the Composition of Shell Cross-Linked Amphiphilic Core−Shell Nanoparticles and the Partitioning of Sequestered Fluorinated Guests

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ABSTRACT: Rotational-echo double-resonance (REDOR) $^{13}C\{^{19}F\}$ and $^{15}N\{^{19}F\}$ NMR spectra have been obtained for guest−host complexes of 6-fluorotryptophan and 4-(trifluoromethyl)benzophenone with polymeric amphiphilic nanoparticles, consisting of a polystyrene core covalently attached to a poly(acrylic acid)-*co*-[^{15}N]polyacrylamide shell. The REDOR spectra were used both to determine the composition of the nanoparticles and to find the locations of the fluorinated guests. Each guest resides near the core−shell interface of the nanoparticle, with the hydrophobic 4-(trifluoromethyl)benzophenone within the core and the amphiphilic 6-fluorotryptophan within the shell.

Introduction

The term shell cross-linked knedel (SCK) refers to an amphiphilic nanoparticle with a core−shell morphology.[1] SCKs are formed by cross-linking micelles made from diblock copolymers, to yield mechanically robust and chemically versatile nanostructured materials. The SCK nanoparticles resulting from diamine cross-linking of polystyrene-*b*-poly(acrylic acid) micelles[2,3] have a rigid, hydrophobic polystyrene core and a flexible, hydrophilic poly(acrylic acid)-*co*-polyacrylamide shell.[4] Because of the amphiphilic core−shell morphology and nanometer-scale dimensions, the SCKs are of interest for use as mimics of lipoproteins, serving as compartments for the sequestration of undesirable metabolites.[5] The ability to partition guest molecules within a nanoscopic particle having a uniform cross-linked membrane-like shell is also of interest for controlled delivery of active agents, for instance in medical or environmental applications. The critical parameter in any application that involves the placement of guests within a host is the guest location. In the case of nanoscale particles, accurate determination of guest location is nontrivial—diffraction experiments are not applicable, conventional solution-state NMR provides no help, and the particles cannot be dissected. This paper reports the use of rotational-echo double-resonance[6] (REDOR) $^{13}C\{^{19}F\}$ and $^{15}N\{^{19}F\}$ solid-state NMR to determine the partitioning between the shell and core of host SCKs of two guests: the amphiphilic 6-fluorotryptophan and the hydrophobic 4-(trifluoromethyl)benzophenone.

Experimental Section

Synthesis. The SCKs were prepared from polystyrene$_{130}$-*b*-poly(acrylic acid)$_{120}$ diblock copolymers, which had 130 polystyrene repeat units and 120 poly(acrylic acid) repeat units per chain, organized into polymer micelles in water. The poly(acrylic acid) chain segments located within the shell of the polymer micelles were cross-linked (Scheme 1) with varying amounts of 2,2′-(ethylenedioxy)bis(ethylamine-^{15}N) in the presence of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide methiodide,[2] resulting in the conversion of 14−55% of the acrylic acid functional groups to ^{15}N-labeled amide linkages. These amidation reactions result in varying degrees of mono- and disubstitution of the diamine; Scheme 1 illustrates a special case. The urea byproduct of cross-linking and all noncoupled diamine were removed by dialysis for all samples except that whose spectra appear in Figure 1. The cores of the SCKs are rigid at room temperature because of the high glass-transition temperature of polystyrene (T_g = 105 °C). The diameters of the roughly spherical SCK nanoparticles were 28 nm, as determined by atomic force microscopy.[1,2] Assuming equal packing densities for core and shell, the core diameter is about 20 nm.

SCK host−guest complexes were formed by adding DL-6-fluorotryptophan (10 wt %) to a solution of SCKs in a mixture of water and tetrahydrofuran (20% THF/water) or in water alone. Water swells the poly(acrylic acid)-*co*-polyacrylamide shell, and tetrahydrofuran solvates the polystyrene core so that the 6-fluorotryptophan in THF/water was complexed under conditions that potentially allowed for loading throughout the SCK. After 12 h, the THF was removed by evaporation under vacuum. The remaining aqueous solution of the 6-fluorotryptophan−SCK complex was frozen rapidly, and the water was removed by lyophilization; 6-fluorotryptophan complexes without THF were made similarly, but without the evaporation step. SCK complexes with 4-(trifluoromethyl)benzophenone were lyophilized from 5 wt % solutions in water. The resulting powders were packed into 7.5 mm o.d. zirconia rotors for examination by solid-state NMR.

REDOR NMR. $^{13}C\{^{15}N$ or $^{19}F\}$ REDOR was performed using a six-frequency transmission-line probe having a 12 mm long, 6 mm i.d. analytical coil and a Chemagnetics/Varian ceramic stator. Powdered SCK samples were contained in thin-wall Chemagnetics/Varian 5 mm o.d. zirconia rotors. The rotors were spun at 6250 or 7143 Hz with the speed under active control to within ±2 Hz. The spectrometer was controlled by a Tecmag pulse programmer. ^{13}C radio-frequency pulses (125 MHz) and ^{15}N radio-frequency pulses (50.7 MHz) were produced by 1 and 2 kW American Microwave Technology power amplifiers, respectively. ^{1}H (500 MHz) and ^{19}F (470 MHz) radio-frequency pulses were generated by 1 kW Creative Electronics tube amplifiers driven by 50 W American Microwave Technology power amplifiers. The π pulse lengths were 10 μs for ^{13}C and ^{15}N and 5 μs for ^{19}F. Distance measurements using ^{19}F dephasing were calibrated using the two-bond coupling of [^{19}F]polycarbonate as described previously.[7] Standard XY-8 phase cycling was used for REDOR. A 12 T static magnetic field was provided by an 89 mm bore Magnex superconducting solenoid. Proton-carbon cross-polarization transfers were made in 2 ms at 50 kHz. Proton dipolar decoupling was 100 kHz during data acquisition. Cross-polarization magic-angle spinning ^{13}C NMR spectra of an SCK

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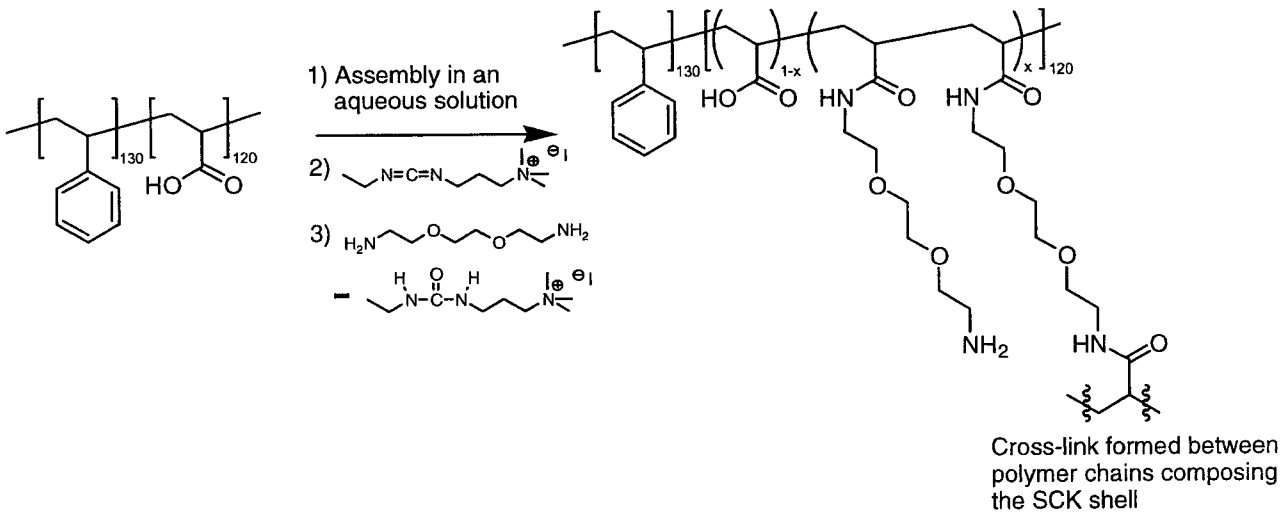
Scheme 1



with a plasticized shell were obtained with a spectrometer operating at 4.7 T, as described earlier.[5]

Results and Discussion

Plasticized Shell. Carbon resonances uniquely identified with the SCK core (nonprotonated aromatic carbon, 145 ppm), shell (carbonyl carbon, 180 ppm; oxygenated carbon, 70 ppm), and 6-fluorotryptophan guest (ring carbons, 95 ppm) are observed by magic-angle spinning (Figure 1). The methyl-carbon peak of the trimethylamine group of the urea byproduct of cross-linking is also resolved (15 ppm). If this component is not removed by dialysis, the shell of the SCK becomes plasticized. The core and shell are now phase-separated with distinctly different relaxation properties. The core has an approximately 10 ms $T_{1\rho}$(H), characteristic of glassy polystyrene at 300 K. The $T_{1\rho}$(H) of the shell, the plasticizer, and 6-fluorotryptophan, by contrast, are all reduced to about 1 ms. After a long proton spin lock in a proton-carbon cross-polarization transfer, only the polystyrene core resonances remain (Figure 1, top). This result shows that the plasticizer is excluded from the core and is restricted to the shell. Carbon signals from the guest 6-fluorotryptophan are also suppressed by the long proton spin lock, indicating dipolar contact between the protons of the guest and those of the shell. This behavior is only consistent with location of the 6-fluorotryptophan guest exclusively in the plasticized shell.

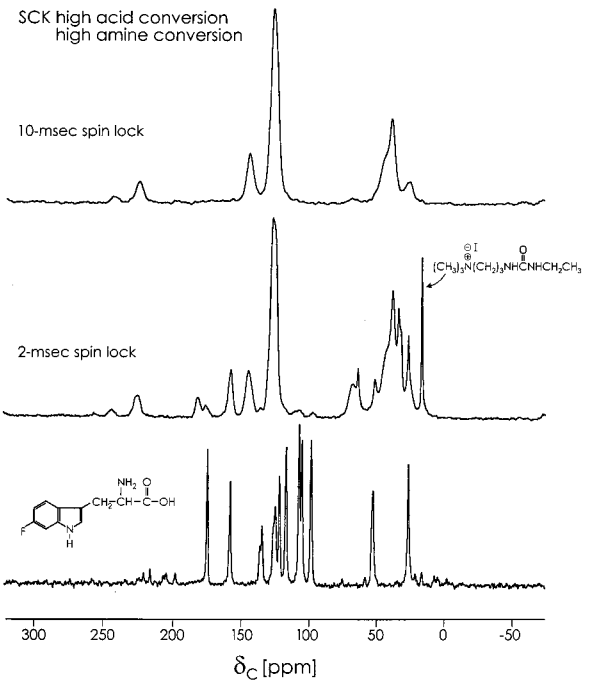


Figure 1. The 50.3 MHz cross-polarization magic-angle spinning ^{13}C NMR spectra of 6-fluorotryptophan sequestered in an SCK after matched ^{1}H−^{13}C spin locks of 10 ms (top) and 2 ms (middle). The cross-linked shell contains trimethylamino groups as part of a urea byproduct of the cross-linking reaction, which shortens the $T_{1\rho}$(H) of the shell relative to that of the core. Only carbon resonances of the polystyrene core are observed after the long spin lock. A reference spectrum of 6-fluorotryptophan (obtained with simultaneous 100 kHz proton and fluorine decoupling) is shown at the bottom of the figure. Magic-angle spinning was at 5 kHz.

Compositional Analysis. Many peaks in the SCK carbon spectrum are combination lines complicating a compositional analysis. For example, the mainchain methine and methylene carbons of both the polystyrene core and the poly(acrylic acid) shell contribute to the collection of peaks centered at 40 ppm. We illustrate the various possibilities for compositional analysis using the ^{13}C{^{15}N} REDOR spectra of a linear poly(acrylic acid), partially cross-linked by $^{15}N_2$-labeled diamine (Figure 2). The 180 ppm region has contributions from both carboxyl and amide carbonyl carbons (comparison 1 in Figure 2); however, only the latter gives rise to a REDOR difference (comparison 2).

The number of amines of the linker converted to amides is established by the ratio of intensities of the REDOR differences at 175 and 40 ppm, respectively (comparison 3). If all the amines are converted to amides, this ratio is one, neglecting differences due to T_2's, which are small for the $8T_r$ evolution time. Of course, the fraction of converted amines is also available from a direct comparison of the amide and amine-nitrogen intensities in the ^{15}N NMR spectrum (cf. below). This fraction does not necessarily reflect the *inter*chain cross-link density because the diamine can connect two carboxyl groups of the same chain. Nevertheless, for the remainder of this paper, we will assume that there is a direct connection between amide-bond formation and cross-link density. The total number of diamine linkers added is determined by the integrated intensity of the 70 ppm oxygenated-carbon peak to that of the 40 ppm main-chain-carbon peak (comparison 4),

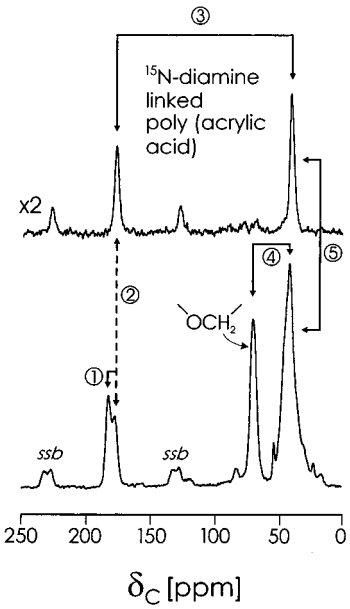


Figure 2. The 125 MHz ^{13}C{^{15}N} REDOR spectra of linear poly(acrylic acid), partially cross-linked by ^{15}N-labeled diamine, after eight rotor cycles of dipolar evolution with magic-angle spinning at 6250 Hz. REDOR difference spectra ($\Delta S = S_0 - S$, where S and S_0 are echo signal intensities with and without dephasing pulses, respectively) are at the top of the figure and full-echo spectra (S_0) at the bottom of the figure. REDOR dephasing is close to complete for directly bonded ^{13}C−^{15}N pairs. Compositional analysis is done using the five indicated intensity comparisons.

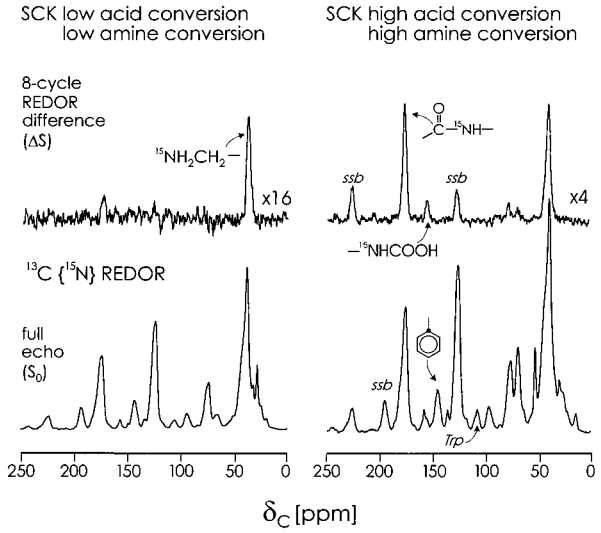


Figure 3. The 125 MHz ^{13}C{^{15}N} REDOR spectra of SCK sample B (left) and sample A (right) of Table 1, after eight rotor cycles of dipolar evolution with magic-angle spinning at 6250 Hz. Compositional analysis of the extent of acid conversion and amine conversion in the cross-linking of the shell by ^{15}N-labeled diamine is made using intensity comparisons 1−5 of Figure 2 (see text). Lyophilization conditions for sample A resulted in carbamylation of about 10% of the amine nitrogens (top right) by atmospheric CO_2 (see ref 8).

once the contribution to the latter from the nitrogenated carbons of the linker has been taken into account (comparison 5).

We use primarily comparison 4 of Figure 2 for the analysis of partially cross-linked SCKs (Figure 3). We measure the extent of diamine substitution of the poly(acrylic acid) shell[8] by n_{linker}: the ratio of nitrogen to carbonyl carbon. This quantity is, in turn, determined

Table 1. NMR Parameters for SCKs of Varying Composition

	F-Trp				CF_3-BP
	THF/water		water		water
sample	A	B	C	D	E
ratio of nitrogen to carbonyl carbon[a]	0.55	0.14	0.53	0.15	0.15
% amine conversion[b]	96	21	92	97	99
^{13}C{^{19}F} 64-T_r $\Delta S/S_0$[c]	0.12	0.11	0.01	0.09	0.06
^{15}N{^{19}F} 64-T_r $\Delta S/S_0$[d]	0.10	0.10	0.03	0.11	0.01
guest location	shell	shell	surface	shell	core

[a] From ratio of ^{13}C intensities at 70 and 40 ppm. [b] From ratio of ^{15}N intensities at 100 ppm (amide) and 10 ppm (amine). [c] For 130 ppm aromatic-carbon peaks. [d] For both 100 ppm (amide) and 10 ppm (amine) nitrogen peaks.

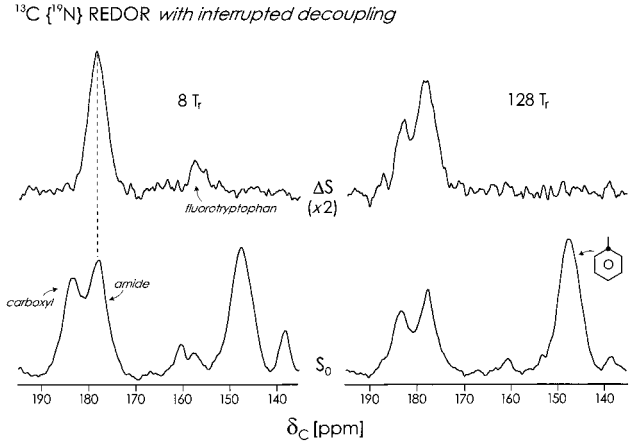


Figure 4. The 125 MHz ^{13}C{^{15}N} REDOR spectra of SCK sample A of Table 1 after 8 (left) and 128 (right) rotor cycles of dipolar evolution with magic-angle spinning at 7143 Hz. The dipolar evolution also included 100 μs of interrupted proton decoupling so that all protonated-carbon signals are suppressed. This removes the interference of the aromatic-carbon spinning sideband from the 180 ppm carbonyl-carbon region. Carboxyl carbons in the shell are sufficiently near an ^{15}N label (within 6 Å) to dephase after 128 rotor cycles of dipolar evolution (right), whereas the nonprotonated aromatic carbons of the polystyrene core are not. The short T_2 of the 158 ppm aromatic-carbon peak of fluorotryptophan prevents detection of detection of dephasing after 128T_r.

by the ratio, r, of the integrated intensity of the 70 ppm peak to that of the 40 ppm peak. As mentioned above, only the oxygenated carbons of the linker contribute to the 70 ppm peak, whereas the main-chain carbons of both the shell and core, as well as the nitrogenated carbons of the linker, contribute to the 40 ppm peak. To a good approximation, there are equal numbers of shell and core main-chain carbons for the SCKs of this study. Thus, $r = 2n_{linker}/(4 + n_{linker})$, which means that $n_{linker} = 4r/(2 - r)$. The value of n_{linker} varies from 0 (for no diamine addition to the shell) to 2 (for maximum substitution). The observed values for n_{linker} for the SCKs of Table 1 range from 0.14 for a lightly cross-linked shell (sample B) to 0.55 for a more densely cross-linked material (sample A). Values of the product of n_{linker} and the percentage of amine conversion to amide (Table 1) not exceeding about 0.5 show that even the most fully substituted and amidated SCKs have about half of all shell side chains carrying carboxyl carbons (Figure 4, left). A complete compositional analysis of a diamine cross-linked polystyrene−poly(acrylic acid) SCK requires the use of all five comparisons of Figure 2. Improvements in the accuracy of measuring integrated peak intensities over that cited in Table 1 are possible

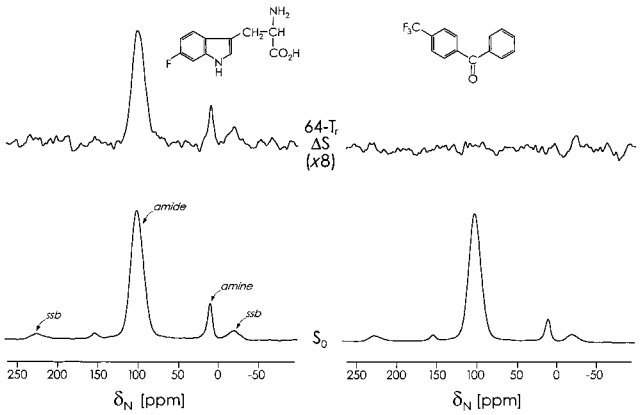


Figure 5. The 50.7 MHz $^{15}N\{^{19}F\}$ REDOR spectra of SCK sample D (left) and sample E (right) of Table 1 after 64 rotor cycles of dipolar evolution with magic-angle spinning at 6250 Hz. Only the complex with 6-fluorotryptophan as guest has a sizable REDOR difference signal (top left). The fraction of amines converted to amides in the SCK shell by cross-linking is determined by the direct comparison of the amide and amine-nitrogen intensities in the full-echo spectra. None of the free amines have been carbamylated (see caption to Figure 3).

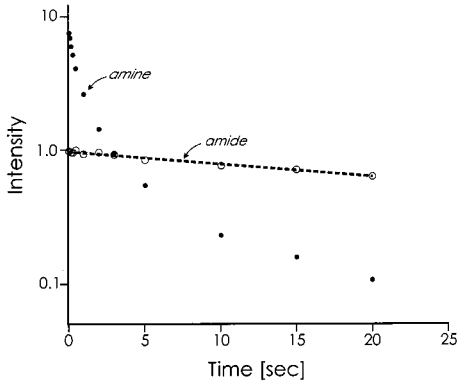


Figure 6. Nitrogen magnetization of SCK sample B of Table 1 as a function of T_1(N) delay.

Table 2. REDOR Dephasing for an SCK with Low Acid Conversion and Low Amine Conversion[a]

	64-T_r $^{15}N\{^{19}F\}$ $\Delta S/S_0$	
	amide	amine
T_1(N) delay, ms[b]		
50	0.14	0.11
300	0.15	0.11
2000	0.17	0.11
$T_{1\rho}$(N) delay, ms[c]		
0.1	0.14	0.11
15	0.14	0.11

[a] Sample B of Table 1. [b] Length of nitrogen magnetization storage along the static field prior to the start of the REDOR evolution period. [c] Length of a nitrogen 42 kHz spin-lock without cross-polarization contact with the protons prior to the start of the REDOR evolution period. The amide S_0 values after spin locks of 0.1 and 15 ms were 1.00 and 0.62, respectively. The corresponding amine S_0 values were 1.00 and 0.65, respectively.

in cross-polarization experiments if rotating-frame relaxation parameters are included using well-established protocols.[9]

Location of Guests. We have previously used $^{13}C\{^{19}F\}$ REDOR dephasing of core- and shell-carbon magnetization to demonstrate[5] that 6-fluorotryptophan loaded from THF/water is at the core−shell interface of a heavily cross-linked SCK (whose composition is similar to that of sample A). Full chemical shift ^{19}F spinning sideband patterns were observed for all the samples of Table 1 (spectra not shown). This indicates the absence of motion of the guests and validity of a direct translation of dipolar couplings into internuclear distances. 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 interface.[5] The REDOR dephasing for 6-fluorotryptophan loaded from THF/water into a lightly cross-linked SCK (sample B, Table 1) is indistinguishable from that for the heavily cross-linked SCK, indicating the same location of the guest on the shell side of the core−shell interface.

Fluorinated guests taken up by SCKs from water alone were trapped by quick freezing and locked into place by lyophilization.[9] For a lightly cross-linked SCK (sample D), the $^{13}C\{^{19}F\}$ and $^{15}N\{^{19}F\}$ REDOR dephasing of $\Delta S/S_0 = 0.09$ and 0.11 (Table 1) for core carbons and shell nitrogens, respectively, indicate that 6-fluorotryptophan reaches the core−shell interface even without THF or the plasticizer of Figure 1. The 6-fluorotryptophan guest must also be primarily, if not exclusively, on the shell side of the interface to account for the more than 50% dephasing observed for the nonprotonated 158 ppm aromatic-carbon peak after only eight rotor cycles of dipolar evolution (Figure 4, left). 6-Fluorotryptophan apparently has no preference for cross-linked or non-cross-linked sites within the shell because the $^{15}N\{^{19}F\}$ dephasing of amide and amine-nitrogen peaks is comparable (Figure 5, left, and Table 1).

6-Fluorotryptophan is unable to penetrate a more highly cross-linked shell (sample C), based on the absence of significant REDOR dephasing of any kind for this system (Table 1). In addition, a 4 ppm downfield isotropic ^{19}F shift was observed for sample C, consistent with the exclusion of 6-fluorotryptophan from the shell to the surface.

For sequestered 4-(trifluoromethyl)benzophenone (sample E), the detection of $^{13}C\{^{19}F\}$ dephasing of aromatic-carbon core peaks (Table 1), but the absence of any significant $^{15}N\{^{19}F\}$dephasing (Figure 5, right), proves that this hydrophobic guest has penetrated the core of a relatively lightly cross-linked SCK. However, the lack of more complete $^{13}C\{^{19}F\}$ dephasing (only 6% after 64 rotor cycles) suggests that the hydrophobic guest is primarily near the interface and is not uniformly distributed throughout the core.[5]

Dynamic Heterogeneity of the SCK Shell. Nonlinear semilog plots of rare-spin magnetization in spin−lattice relaxation measurements are an unambiguous indication of the presence of a distribution of relaxation rates.[10] We interpret the pronounced nonlinearity of the SCK amine-nitrogen T_1 behavior (Figure 6) as due to variations in relaxation arising from local variations in chain packing. Similar relaxation behavior was observed for samples that were heavily cross-linked (high acid conversion, high amine conversion) or lightly cross-linked (low acid conversion, low amine conversion). The relaxation behavior is therefore not the result of the presence of unreacted diamine. The limited extent of the $^{15}N\{^{19}F\}$ REDOR dephasing by 6-fluorotryptophan in samples B and D (Table 1) indicates that only a fraction of the labeled nitrogens of these two SCKs are near 6-fluorotryptophan at the core−shell interface; in other words, the amine ^{15}N labels are located throughout the shell. However, there is no indication that the

heterogeneity in amine-nitrogen T_1 relaxation rates is the result of presumably tighter packing of chains at the interface, which is crowded by the presence of all the 6-fluorotryptophan. Both fast- and slow-relaxing components of the amine-nitrogen population are equally likely to be at the core−shell interface, based on the absence of a dependence of the $^{15}N\{^{19}F\}$ REDOR dephasing on T_1 or $T_{1\rho}$ preparation times (Table 2).

Conclusions

Solid-state NMR experiments have enabled a detailed analysis of the composition and structure of amphiphilic core−shell SCK nanoparticles. Because the cross-linked shell layer controls the uptake and release of guest species, the cross-link density and cross-link placement are parameters of particular importance. Some insight into these features was provided by combined stable-isotope labeling and solid-state REDOR NMR; more elaborate labeling strategies will be necessary in the future to distinguish quantitatively between the interchain cross-links and the intrachain covalent couplings. The observations of amphiphilic guests migrating to the core−shell interface, and hydrophobic guests entering the core domain, have already been used to guide synthetic efforts to tailor the SCK as a nanoscale containment device.[11]

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