Association of Peptide Chains during Merrifield Solid-Phase Peptide Synthesis. A Deuterium NMR Study

Adriane G. Ludwick,[†§] Lynn W. Jelinski,*[†] David Live,[‡] Agustin Kintanar,[†⊥] and Joseph J. Dumais[†]

Contribution from AT&T Bell Laboratories, Murray Hill, New Jersey 07974, and the Department of Chemistry, Emery University, Atlanta, Georgia 30322. *Received April 7, 1986*

Abstract: Solid-state deuterium NMR spectra are reported for a series of swollen Merrifield resins containing protected glycine oligomers, with the goal of delineating molecular-level interactions that can affect desired reactivity of these materials. In a generic sense, the polystyrene matrix with its pendant glycine oligomers is a comb-type graft copolymer and as such affords a highly controlled model for these polymer systems. Our results are consistent with a model in which partical aggregation of the glycine oligomers occurs after the pendant chain reaches a critical length ($n > 5$). Lengths greater than this correspond to the overlap necessary to form at least one helix repeat of the polyglycine II structure. The polystyrene matrix is concomitantly immobilized, presumably due to additional effective cross-links caused by the aggregation.

Solid-phase peptide synthesis, pioneered by Merrifield in the 1960s,[1] has become a widely accepted method of peptide synthesis and has been modified for oligonucleotide synthesis.[2] It has had a major impact on modern biology.[3] The method involves repeated sequential coupling of residues to a growing carboxyl terminal section of a peptide attached to a lightly cross-linked and therefore macroscopically insoluble polymer matrix, usually 1% divinylbenzene cross-linked polystyrene. While attached to the polymer matrix, the growing peptide is readily recoverable by filtration of the peptide–resin beads. This aspect of the method and the repetitive, stepwise nature of the chemistry lend it to automation.

The chemistry related to optimal incorporation of amino acids has been carefully and extensively investigated.[4] Couplings considered difficult and/or of low yield sometimes occur with particular sequences. These difficulties have been variously attributed to association complexes, swelling problems, or aggregation of the growing peptide chains.[3,5] In contrast to the extensive attention afforded the coupling chemistry, the molecular-level details relevant to mobility of the polymer matrix and of the growing peptide chain that can affect reactivity have only recently been addressed.[6] The view of the role of the support in solid-phase peptide synthesis has been subject to various interpretations. Although it had been assumed that the support isolated the growing peptide chains, creating chemically dilute microenvironments[7], it has now been shown that site–site reactions between different chains do occur[8] and can in fact be made to occur in high yield.[9] Through swelling experiments it has also been demonstrated that during solid-phase peptide synthesis, the peptide chains and the polystyrene support exert a complementary solubilizing effect on each other.[10] An improved understanding of resin and peptide properties is essential in view of the rapidly increasing application of solid-phase synthesis[3] and the desire to efficiently produce even larger polypeptides.

Both solid-state and solution-state deuterium NMR techniques have been used to investigate the interactions between large and small molecules. For example, Jelinski and co-workers have used deuterium NMR to investigate the nature of the water–epoxy interaction[11] and the trapping of water by oxidized polyethylene.[12] In another work, Neurohr and co-workers have examined the dynamics between lectins (wheat germ agglutinin) and deuterium-labeled *N*-acetylglucosamines.[13] In these and other investigations it is evident that deuterium NMR is a powerful spectroscopic tool, capable of revealing significant details of molecular-level interactions in complex systems.[14]

We report here a quadrupole echo deuterium NMR study that directly addresses the questions of molecular motion (and thereby accessibility) of both the swollen polystyrene matrix and the growing glycine oligomers in the following series of samples:

Table I. Characterization of Polystyrene Resin–(Glycine-d_2)$_n$ Samples

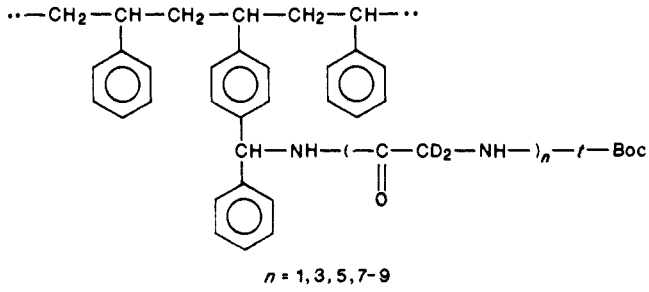
no.	gly residues	sample % completion	R
1	1	99.5[a]	gly-d_2-Boc
2	3	99.8[b]	(gly-d_2)$_3$-Boc
3	5	99.8[b]	(gly-d_2)$_5$-Boc
4	7	99.3[b]	(gly-d_2)$_7$-Boc
5	8	98.7[b]	(gly-d_2)$_8$-Boc
6	9[c]	98.7[b]	(gly-d_2)$_8$gly-Boc
7	9[c]	98.7[b]	(gly-d_2)$_9$-Boc

[a] Completion was monitored by using the picrate method.[17] [b] Completion was monitored by using the ninhydrin test.[16] [c] In sample 6, the terminal glycine was not deuterated; in sample 7, all glycines were deuterated.

[†] AT&T Bell Laboratories.
[‡] Emery University.
[§] Participant in AT&T Bell Laboratories Aid to Black Colleges Program. Current address: Department of Chemistry, Tuskegee University, Tuskegee, AL 36088.
[⊥] Current address: Department of Chemistry, University of Washington, Seattle, WA 98195.

(1) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149. (b) Merrifield, R. B. *Science (Washington, D. C.)* **1986**, *232*, 341.
(2) Caruthers, M. *Science (Washington, D. C.)* **1985**, *230*, 281.
(3) Kent, S.; Clark-Lewis, I. In *Synthetic Peptides in Biology and Medicine*; Alitalo, K., Partanen, P., Vaheri, A., Eds.; Elsevier: Amsterdam, 1986.
(4) Barany, G.; Merrifield, R. B. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic: New York, 1974; Vol. 2, pp 1–284.
(5) Merrifield, R. B. *Br. Polym. J.* **1984**, *16*, 173.
(6) (a) Live, D. H.; Kent, S. B. H. In *Elastomers and Rubber Elasticity*; ACS Symposium Series 193; Mark, J., Lol, J., Eds.; American Chemical Society: Washington, D.C, 1982; pp 501–515. (b) Live, D. H.; Kent, S. B. H. In *Peptides: Structure and Function*; Hruby, V., Rich, D., Eds.; Pierce Chemical Co.: Rockford, IL, 1983; pp 65–68.
(7) Leznoff, C. C. *Chem. Soc. Rev.* **1974**, *3*, 65 and references cited.
(8) (a) Farrall, M. J.; Frechet, J. M. J. *J. Am. Chem. Soc.* **1978**, *100*, 7998 and references cited. (b) Rebek, J.; Trend, J. E. *J. Am. Chem. Soc.* **1979**, *101*, 737 and references cited.
(9) Bhargava, K. K.; Sarin, V. K.; Trang, N. L.; Cerami, A.; Merrifield, R. B. *J. Am. Chem. Soc.* **1983**, *105*, 3247.
(10) Sarin, V. K.; Kent, S. B. H.; Merrifield, R. B. *J. Am. Chem. Soc.* **1980**, *102*, 5463.
(11) (a) Jelinski, L. W.; Dumais, J. J.; Cholli, A. L.; Ellis, T. S.; Karasz, F. E. *Macromolecules* **1985**, *18*, 1091. (b) Jelinski, L. W.; Dumais, J. J.; Stark, R. E.; Ellis, T. S.; Karasz, F. E. *Macromolecues* **1983**, *16*, 1019.
(12) McCall, D. W.; Douglass, D. C.; Blyler, L. L., Jr.; Johnson, G. E.; Jelinski, L. W.; Bair, H. E. *Macromolecules* **1984**, *17*, 1644.
(13) Neurohr, K. J.; Lacelle, N.; Mantsch, H. H.; Smith, I. C. P. *Biophys. J.* **1980**, *32*, 931.
(14) Jelinski, L. W. *Adv. Mater. Sci.* **1985**, *15*, 359.

0002-7863/86/1508-6493$01.50/0



In a generic sense, the polystyrene matrix with its pendant glycine oligomers is a comb-type graft copolymer and as such affords a highly controlled model for these polymer systems. In particular, the polystyrene phase and the polyglycine phase are not compatible, driving the system toward microphase separation.

Experimental Section

The resin–peptides used in this study were prepared on a Schwartz/Mann automated peptide synthesizer using *t*-Boc-gly-d_2. One percent cross-linked polystyrene with 0.5 mequiv/g of benzhydrylamine groups (Beckman) was used following procedures reported elsewhere.[15] Methylene chloride was the solvent during coupling. Couplings were performed for 1 h using a 2-fold excess of Boc-gly-d_2 for samples **1–5** and a 3-fold excess for samples **6** and **7**. Loading of the first amino acid on the resin was determined by hydrolysis and amino acid analysis. Completion of coupling was monitored after each coupling cycle by using either the ninhydrin[16] or picrate[17] method. Sample designations, sample composition, and percent completions are summarized in Table I. This table shows that the couplings essentially went to completion until after five glycine residues had been added.

The degree of swelling of these resins in methylene chloride and dimethylformamide was estimated by placing a known amount of resin in a 5-mm NMR tube and adding excess solvent. The height of the resin was compared to that of fully collapsed resin in methanol. These results are summarized in Table II.

Deuterium NMR spectra were obtained at 55.26 MHz by using the quadrupole echo pulse sequence.[18] The spectrometer has been described previously.[19] The digitization rate for the powdered samples was 100 ns/point (10 MHz), while for the DMF-swollen samples the digitization rate was 1 μs/point (1 MHz). The 90° pulse width was 3.1 μs. Solution-state carbon spectra of both DMF and methylene chloride swollen resins were obtained at 75.46 MHz on a Nicolet spectrometer. Generally 1000 accumulations were obtained by using a 20-kHz spectral window.

It is important to note that rapid digitization, high power radio frequency pulses, and the quadrupole echo pulse sequence were essential for obtaining the combination broad-line/narrow-line spectra reported here. Temperature and recycle delay times are reported in the figure captions.

Results and Discussion

The deuterium NMR spectra of all of the solid-phase peptide samples in the powdered, unswollen solid state were observed with a 2-s recycle rate, and all showed the Pake line shape expected for a static C–D bond[14] both at ambient temperature (23 °C) and at elevated temperatures (101–112 °C). Representative spectra of sample **7** at ambient and elevated temperatures are shown as examples in Figure 1. These spectra indicate that in the unswollen solid state, the glycine groups bonded to the polystyrene matrix are static on the time scale of the deuterium NMR experiment. Additionally, heating the solid samples to approximately 100 °C does not appear to alter the motional dynamics of these systems. These results are not unexpected for a solid copolymer system and indicate that the large-amplitude motions with characteristic frequencies greater than several kilohertz are induced only by swelling the sample (vide infra). Shown for comparison in Figure 2 are the corresponding solid-state NMR spectra for the polycrystalline amino acid, glycine-d_2. These spectra also show a static Pake powder spectrum, as expected. It is a significant observation

Table II. Swelling of Resin–(Glycin-d_2)$_n$ Samples: Height of Swollen Resin (cm) in a Tube for a Constant Weight of Peptide–Resin

no.	CH_2Cl_2	DMF	CH_3OH (collapsed)
2	2	3	1.2–1.3
3	1.3	2.85	1.2–1.3
4	1.3	1.5	1.2–1.3
7	1.3	1.4	1.2–1.3

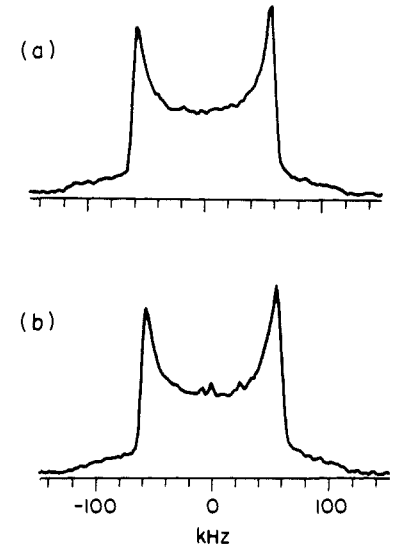


Figure 1. Quadrupole echo deuterium NMR spectra at (a) 23 °C and (b) 101 °C obtained with a 2-s recycle delay. All samples in Table I produce similar results.

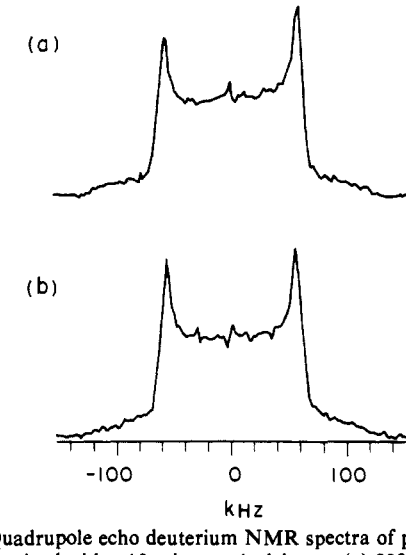


Figure 2. Quadrupole echo deuterium NMR spectra of polycrystalline glycine-d_2 obtained with a 10-min recycle delay, at (a) 23°C and (b) 100 °C.

(15) Live, D.; Agosta, W. C.; Cowburn, D. *J. Org. Chem.* **1977**, *42*, 3556.
(16) Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.* **1981**, *117*, 147.
(17) (a) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248. (b) Hodges, R. S.; Merrifield, R. B. *Anal. Biochem.* **1975**, *65*, 241.
(18) (a) Davis, J. H.; Jeffrey, K. R.; Bloom, M.; Valic, M. I.; Higgs, T. P. *Chem. Phys. Lett.* **1976**, *42*, 390. (b) Blinc, R.; Rutar, V.; Seliger, J.; Slak, J.; Smolej, V. *Chem. Phys. Lett.* **1977**, *48*, 576. (c) Hentschel, R.; Spiess, H. W. *J. Magn. Reson.* **1979**, *35*, 157.
(19) (a) Jelinski, L. W.; Dumais, J. J.; Engel, A. K. *Macromolecules* **1983**, *16*, 492. (b) Cholli, A. L.; Dumais, J. J.; Engel, A. K.; Jelinski, L. W. *Macromolecules* **1984**, *17*, 2399.
(20) Merrifield, R. B.; Littau, V. In *Peptides 1968*; Bricas, E., Ed.; North Holland: Amsterdam, 1968; p 179.
(21) Tonelli, A. E. In *Encyclopedia of Polymer Science and Engineering*, 2nd ed.; Wiley: New York, 1985; Vol. 4.
(22) Elias, H.-G. *Macromolecules*; Plenum: New York, 1984; Vol. 1, pp 119–121.
(23) Flory, P. J. *Statistical Mechanics of Chain Molecules*, Interscience: New York, 1969; p 149.
(24) Ramachandran, G. N.; Ramakrishnan, C.; Venkatachalam, C. M. In *Conformation of Biopolymers*; Ramachandran, G. N., Ed.; Academic: New York, 1967; Vol. 2, pp 429–438.
(25) Padden, F. J., Jr.; Keith, H. D. *J. Appl. Phys.* **1965**, *36*, 2987.
(26) Narita, M.; Isokawa, S.; Tomotake, Y.; Nagasawa, S. *Polym. J.* **1983**, *15*, 25.

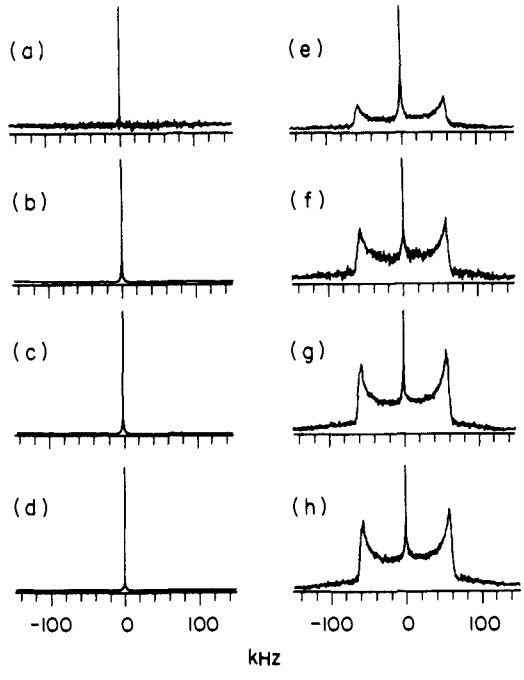


Figure 3. Quadrupole echo deuterium NMR spectra of DMF swollen materials for (a) the polystyrene matrix and for solid-phase peptide samples (b) **1** (gly-d_2), (c) **2** ((gly-d_2)$_3$), (d) **3** ((gly-d_2)$_5$), (e) **4** ((gly-d_2)$_7$), (f) **5** ((gly-d_2)$_8$), (g) **6** ((gly-d_2)$_8$gly), and (h) **7** ((gly-d_2)$_9$). The spectra were obtained with a recycle delay time of 2 s.

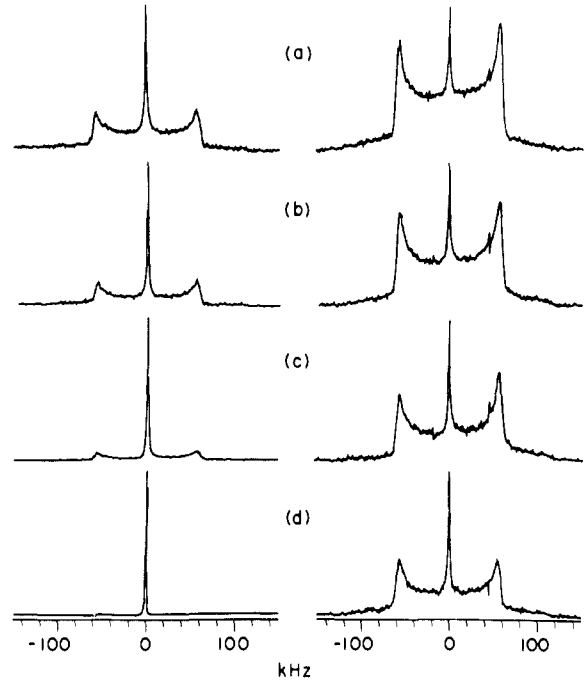


Figure 4. Quadrupole echo deuterium NMR spectra of DMF swollen sample **4** (left column) and DMF swollen sample **7** (right column) obtained at (a) 23, (b) 50, (c) 82, and (d) 111 °C.

that it was necessary to use a recycle delay time of 10 min to obtain fully relaxed spectra of polycrystalline glycine, whereas the deuterated glycine oligomers on the polystyrene backbone required a recycle time of less than 2 s. This indicates that the amorphous, glassy polymeric matrix exhibits its influence on the growing peptide chains, even in the dry, solid state.

When the solid-phase peptide samples were swollen in dimethylformamide (DMF), it became possible to distinguish the samples of Table I spectroscopically. In the swollen state, the line shape indicative of a static C–D bond is not observed in the deuterium NMR spectra of samples **1–3** (corresponding to Figure 3b–3d, respectively). The sharp line observed for these samples arises because the glycine residues in the swollen state are sufficiently mobile to undergo isotropic or nearly isotropic motion. A small amount of this sharp signal arises from naturally abundant deuterium in the solvent. However, the static pattern becomes increasingly evident as the chain length of the glycine oligomer pendant to the chain increases (samples **4–7** corresponding to Figure 3e–3h, respectively). To determine the dependence of the observations in Figure 3 on the weight percent of solvent in the sample, this ratio was varied so that the sample was either freely flowing or highly viscous. Additionally, the length of time the sample was exposed to solvent was varied from 12 to 36 h. No significant differences in the observations of Figure 3 were noted with these variations.

The sharp component of the line shapes in Figure 3f–3h (samples **5–7**, respectively) is due primarily to the natural abundance deuterium signal from the solvent. When the signal from the solvent is subtracted out, the spectra for samples **5–7** reveal a small amount of a broader but nearly isotropic signal that comprises approximately 2% of the total integrated intensity. It is likely that this signal arises from the small amount of shorter sequences that are present as a consequence of imperfect coupling (see Table I) or noninteracting chains. In contrast, sample **4** (the spectrum in Figure 3e) contains approximately 15% of its intensity in the nearly isotropic signal that arises from the glycine oligomers.

Heating samples **4** and **7** to 111 °C resulted in significant decreases in the static portions of the spectra (Figure 4). Cooling the samples to room temperature resulted in recovery of most of the static portion for sample **4** and all of the static portion for sample **7**. Holding sample **4** at room temperature for 24 h did not significantly increase this percent recovery, although long-term annealing did.

The deuterium NMR results for the swollen polymer resin show an increase in the fraction of glycine units contributing to the static spectrum as the number of amino acids is increased. The critical number of glycine units is apparently greater than five and perhaps close to seven (see Figure 3e). In a related study, solution-state ^{13}C NMR spectra (not shown here) were obtained at 75.46 MHz for samples **2–4** and **7** in DMF and in methylene chloride. The polystyrene resin (swollen in DMF) exhibited sharp aromatic and aliphatic carbon resonances for samples **2** and **3** but not for **4** and **7**. The sharp resonances observed for samples **2** and **3** under these spectroscopic conditions indicate that the polystyrene matrix is nearly isotropically mobile on the NMR time scale. The loss of ^{13}C signal intensity for samples **4** and **7** is caused by static dipole–dipole and chemical shift anisotropy interactions and implies significant loss of polymer mobility. Recent work by Errede and Newmark[27] has shown that aromatic carbon line widths in swollen polystyrene gels are a linear function of the cross-link density. Taken together, these results indicate that the onset of the static-like line shape observed by quadrupole echo deuterium NMR spectroscopy is accompanied by a concomitant loss in mobility of the polystyrene resin. These results correlate well with the observation that the apparent degree to which the samples swell upon the addition of DMF decreased as the side chain length increased (Table II).

It is observed that the system with nine deuterated glycine residues (sample **7**) appears to be as motionally restricted as the system with eight deuterated glycine residues and one undeuterated glycine residue (sample **6**). Furthermore, a comparison of the spectra for sample **6** with sample **5** (Figure 3g and 3f, respectively) shows that the static signal increases as the chain length increases and not merely as the number of deuterated units present on the chain, since both of these pendant groups have eight deuterated glycines.

The following calculations, as well as inspection of a molecular model of a section of an oligoglycine bearing solid-phase resin, offer a consistent rationale for these observations. On the basis of 0.5 mequiv of glycine/g of polystyrene, it is calculated that the

(27) Errede, L. A.; Newmark, R. A.; Hill, J. R. *Macromolecules* **1986**, *19*, 651.

Table III. Rotational Isomeric State Calculation for Polystyrene

$\langle L^2\rangle^{1/2}$, Å	P_m[a]	$\langle L^2\rangle^{1/2}$, Å	P_m[a]
31.1	0 (syndiotactic)	27.4	0.6
30.2	0.1	28.4	0.7
28.8	0.2	28.8	0.8
28.3	0.3	29.7	0.9
28.3	0.4	31.3	1.0 (isotactic)
27.6	0.5 (atactic)		

[a] Probability of a meso placement.

pendant peptide chains will be separated, on the average,[20] by 18 styrene monomer units.

There are a number of ways to estimate the mean-squared distance between the polystyrene rings that bear the pendant glycine oligomers.[21] For large N, where N is the number of C–C bonds, the mean-squared distance, $\langle L^2\rangle$, between styrene residues that bear oligoglycine chains can be estimated from[22]

$$\langle L^2\rangle = Nb^2\frac{(1+\cos\tau)}{(1-\cos\tau)}\sigma^2$$

where b is the bond length and is taken as 1.54 Å, τ is the supplement of the valence angle (assumed to be tetrahedral), and σ is the steric hindrance parameter and is taken as 2.6.[22] The square root of $\langle L^2\rangle$ is thus estimated as 26.2 Å. This estimate holds only for large N, which for other polymers such as polyethylene is greater than 100.[23]

In this case it is therefore more realistic to perform a rotational isomeric state (RIS) calculation[21] to determine the distance between pendant sites. The results from such a calculation are listed in Table III. $\langle L^2\rangle$ for an atactic polystyrene chain ($P_m = 0.5$) is estimated as 27.6 Å. (P_m is the probability of a meso placement along the chain.)

We now determine the length of the oligoglycine chains. The glycine residues, by virtue of their peptide bonds, are expected to be somewhat more conformationally restricted than the polystyrene backbone. Therefore, a distance of 3.1 Å per glycine unit in the oligoglycine chain is estimated from the structure of polyglycine II.[24] Polyglycine II is known to pack into helixes with a 3-fold screw axis with a repeat distance of 9.3 Å[24] with adjacent chains running in opposite directions.[25] With this structure as a model for the pendant oligoglycines on the resin, it is reasonable to estimate that the oligoglycine chains would be able to overlap, without concomitant distortion of the polymer backbone, by approximately 3.4 Å when the peptide chain is a pentamer. This overlap becomes approximately 15.8 Å at the heptamer length, somewhat greater than the amount of overlap required to form one repeat of the polyglycine II structure.[24] It is at the heptamer level that the quadrupole echo deuterium NMR spectrum first shows evidence of a static-like structure (Figure 3e). These estimates are borne out by inspection of molecular models. It is therefore tempting to attribute the static-like spectra to formation of polyglycine aggregates. Such aggregation of resin-bound oligopeptides has been observed previously by infrared spectroscopy for oligoleucines.[26] The infrared results also show that the resin-bound peptide chains can easily interact with each other through hydrogen-bonding interactions.

A statistical distribution of the glycine oligomers along the backbone will allow the side chains to hydrogen bond to their nearest neighbors to different extents. It will also produce some chains incapable of reaching the nearest pendant group on the backbone and some which form cross-links to other parts of the polymer matrix. The extent of nearest-neighbor and cross-link overlap should be greater for sample **7** than **4**. At high temperatures, groups with the largest degree of overlap will remain associated, while those with lesser overlap would most likely separate. Upon cooling it would be expected that these hydrogen bonds which were broken would return in some time–dependent manner. The slower association of **4** than **7** upon cooling is consistent with this rationale. This separation of pendant chains forming thermally labile cross-links allows the polymer to swell more completely. The percentage of such cross-links would presumably be greater for **4** than **7**. Once these cross-links are broken and additional swelling occurs, cooling the sample may not be sufficient to allow the association of the weaker cross-links to recur immediately, thus accounting for the decreased intensity of the static portion of the spectrum for **4** even after 24 h at room temperature.

This hypothesis was tested by changing the swelling solvent from DMF to methylene chloride, since methylene chloride has been shown to solvate peptides less than DMF.[3] DMF produces better solvated peptides as born out by the finding that the peptide–resins are more poorly swollen in methylene chloride (Table II) and by the reduced mobility of the peptide chains in methylene chloride found from NMR. Inspite of very poor swelling in the latter solvent the couplings proceed surprisingly well (Table I). However, the lower oligomers (i.e., the samples that swell normally) provide NMR results that are similar to those reported previously by Live and Kent.[6]

It should be noted that the system studied here was selected to be an extreme example for promoting inter- and intrachain interactions in the peptide–resin, since the pendant peptide is a homopolymer of a residue with a minumum of steric hindrance. Furthermore, the benzhydrylamine supports possess differences in swelling properties from the simple chloromethylated resins and resins with various extended groups. The results show that even in this circumstance DMF can compete with the oligopeptide interactions. The results argue against the use of peptide-like polyamide supports in solid-phase synthesis since they present the possibility for peptide–support interactions that could be restrictive to the growing peptide.

The foregoing results show that quadrupole echo deuterium NMR spectroscopy can provide detailed, molecular-level information about solid-phase peptide–resins, thereby leading to a better understanding of the influences of various pendant groups on the reactivity of these systems. One could imagine important extensions of these methods to observe molecular dynamics of similarly tethered peptides and proteins in vivo.