Characterizing the structure and dynamics of folded oligomers: Pulsed ESR studies of peptoid helices†

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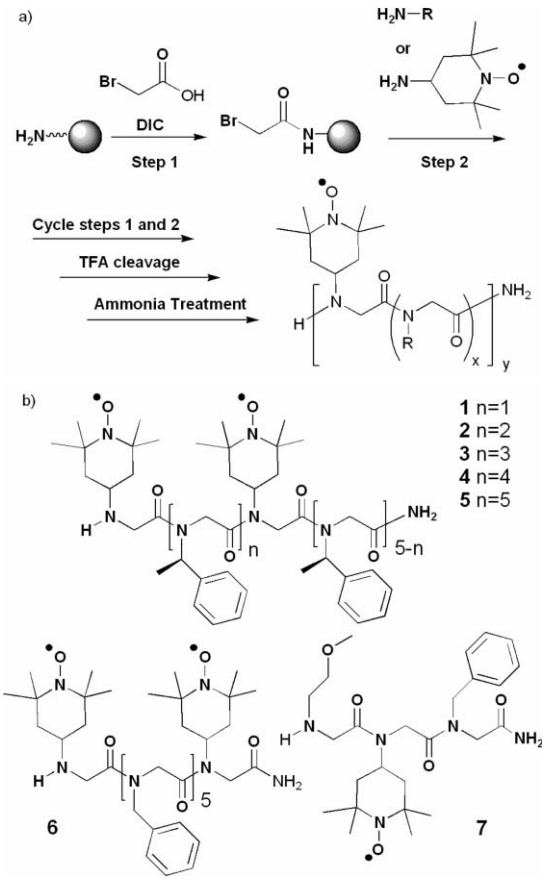
Helical peptoid oligomers were synthesized in which the positions of nitroxide radical spin probes along the backbone were systematically varied, allowing evaluation of intramolecular distances and dynamics by electron spin resonance spectroscopy.

Synthetic oligomers that self-assemble into stable folded conformations have garnered substantial attention due to their biomimetic character.[1] For example, sequence-specific "peptoid" oligomers comprised of diverse N-substituted glycine monomers have been described that display some of the structural and functional attributes of conformationally-ordered polypeptides.[1b] Ongoing studies seek to explore the biomedical applications of peptoids and to enhance our understanding of how peptoids fold. Peptoids incorporating bulky chiral sidechains are known to be capable of forming stable helical structures, even at short chain lengths.[2,3] Further progress in the design of peptoids and other functional oligomers capable of self-directed folding will require the development of methods for characterizing their structure and dynamics.

Paramagnetic spin labels have proven valuable for the study of an array of polymers and biomolecules[4] including proteins,[5] peptides[6] and RNA[7] as well as other synthetic folded oligomers.[8] In particular the 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) group has been employed as it is stable to the conditions of peptide synthesis.[4,9,10] Using electron spin resonance (ESR) techniques applied to compounds containing two nitroxide moieties, it is possible to determine accurately the distance between the probe groups, establishing spatial constraints on the molecular structure.[4,5a,c,d,7b,c,11,12] For the structural characterization of conformationally flexible molecules, techniques such as ESR and fluorescence resonance energy transfer (FRET)[3] are important additions to X-ray crystallography and NMR.[2] ESR and FRET can be applied to the study of dynamic systems and thus have the potential for quantitative evaluation of the behavior of peptoids spanning the regime from stable helices to highly random conformations. ESR methods, by giving distances directly, can readily provide the variance of the distribution of inter-probe distances around their average, and thus indicate the extent to which a molecule has a rigid or flexible structure.[5a,c,12,13] Also, nitroxide labels are significantly less perturbing than fluorescent probes. Here we demonstrate the facile introduction of TEMPO sidechains into peptoid sequences and the subsequent application of ESR to elucidate peptoid structure.

4-Amino-TEMPO was incorporated into a family of peptoid sequences by a straightforward modification of the standard automated "submonomer" protocol for solid phase synthesis (Scheme 1).[14] A series of double-labeled peptoid heptamers was synthesized incorporating one TEMPO group at the N-terminus and a second TEMPO group at varying positions along the chain (compounds **1–6**, Scheme 1(b)). A single-labeled peptoid trimer **7**



Scheme 1 (a) The submonomer synthesis approach for spin-labeling peptoid oligomers. In step 2, 4-amino TEMPO can be used as a synthon to incorporate a stable nitroxide free radical. Aqueous ammonia is required after acid cleavage to regenerate the free radical species. TFA: trifluoroacetic acid; DIC: diisopropylcarbodiimide. (b) Chemical structures of spin labeled peptoids **1–7**.

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was synthesized as a reference compound. TFA cleavage was used to liberate the peptoids from solid phase support, following which aqueous ammonia treatment was required to re-establish the desired nitroxide free radical species.‡

To ascertain whether peptoids incorporating the TEMPO group retained a propensity to form stable helices we utilized circular dichroism (CD) spectroscopy (Fig. 1). The structural motif explored in this report, comprising chiral phenylethyl side-chains, has been studied extensively and it was found previously that these side chains are conducive to the formation of helical secondary structure at chain lengths as short as pentamers.[2] The qualitative CD features and ellipticity values in a representative CD spectrum for double-labeled heptamer **1** (Fig. 1) resemble those generated by peptide α-helices. However, for peptoids containing (*S*)-phenylethyl sidechains, this signature has been associated with a well-ordered polyproline I-like helical secondary structure.[2]

This characteristic CD feature is not perturbed by the presence of the TEMPO side chains in **1–5**, suggesting that the conformation of these peptoids retains the helical secondary structure we seek to characterize.

ESR studies were then conducted on the family of double spin-labeled peptoids. In these experiments, the extent of dipolar coupling provides a direct measure of the distance between two unpaired electron spins. Dipolar coupling interactions can be measured from the static dipolar broadening of continuous wave (CW) ESR spectra[4,5d,11d] or, alternatively, from the influence of coupling on the evolution of coherences in pulsed ESR experiments.[5a,c,6b,c,7b,c,11c] For distances longer than ~15 Å, the use of CW methods is confounded as the small broadenings caused by dipolar couplings are masked by larger static inhomogeneous broadenings arising from anisotropic magnetic tensors of the nitroxides. In contrast, pulsed ESR methods based on detection of electron spin-echoes cancel the effect of inhomogeneous broadenings. For example, double quantum coherence (DQC) ESR methods using intense pulses can be applied to determine distances in the 10–75 Å range.[5a,c,6d,7b]

DQC ESR data were collected for 250 μM methanolic (1% H_2O) solutions of **1–6** using a six-pulse sequence[5a] after flash-freezing at 70 K (*cf.* Fig. 2). A working frequency of 17.4 GHz, with the 30 G magnetic component of the RF field in the rotating frame of reference was adequate for distances of 11 Å or longer. These data provided quantitative distance measurements between the two nitroxide moieties in each compound as well as a

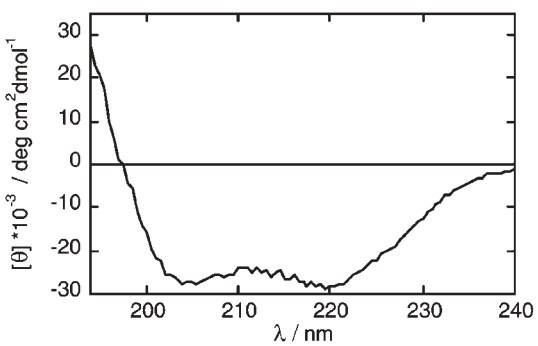

Fig. 1 Representative CD spectrum for double spin-labeled oligomer **1** in acetonitrile (100 μM, 1 mm path length). For peptoids, this signature is indicative of a polyproline-I like helical structure.[2]

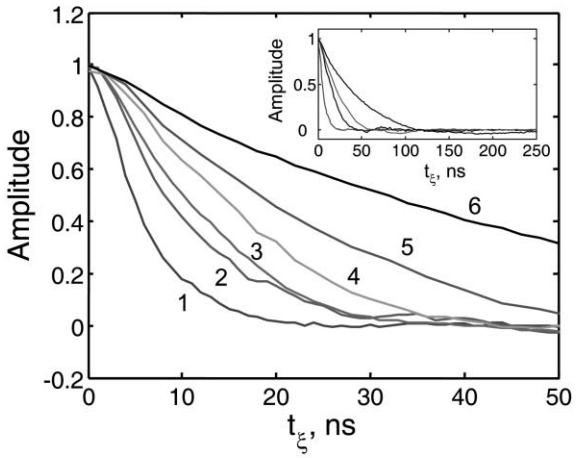

Fig. 2 ESR data for compounds **1–6**. Time domain double quantum coherence (DQC) signal (one half of the symmetric time-domain signal), plotted *vs.* relevant time variable t_ξ of the six-pulse DQC sequence. Inset shows longer time-scale with **2** and **3** omitted for clarity.

comparison of conformational heterogeneity between the compounds (Table 1). The measured distances of **1–5** increase from 12.2 to 18.2 Å, as the inter-residue spacing varies from (*i, i* + 2) to (*i, i* + 6), respectively. From a consideration of the X-ray crystal structure of a similar helical peptoid sequence,[2d] we calculated an anticipated distance of 17 Å for the distance between nitroxide groups in the end-labeled heptamer **5**. This value is within the variance of the DQC determined value of 18.2 Å measured for this compound. The location of a TEMPO group at the N-terminus is anticipated to contribute to the observed variances, as peptoid helices have been shown elsewhere to exhibit some fraying at their termini.[2c]

The extent of conformational heterogeneity could be evaluated through analysis of the distance distributions (Fig. 3) calculated by Tikhonov reconstruction based on the L-curve method.[13] In general, the distributions were fairly narrow for linear oligomers **1–5**, indicative of a well-ordered structure. As expected, the distance distribution was somewhat broadened for compound **5**, in which both probes are located at the weakly constrained termini. Also in accordance with our expectations, the conformational heterogeneity was lower for chiral, structured peptoids in comparison with a similar achiral sequence that does not include structure-inducing phenylethyl sidechains (compare **5** *vs.* **6** in Table 1, Fig. 3).

We conclude that nitroxide free radical spin probes can be efficiently incorporated at multiple positions in synthetic oligomers by utilizing the commercially available 4-amino-TEMPO as a reagent and employing standard "submonomer" solid-phase synthesis protocols. As evidenced by CD spectroscopy, the

Table 1 Mean inter-probe distances and variances of spin-labeled peptoids obtained from distributions measured by DQC (*cf.* Fig. 3)

Compound	Mean distance/Å	Variance/Å
1	12.2	3.4
2	14.5	4.3
3	15.9	4.4
4	17.1	3.3
5	18.2	5.0
6	21.5	6.5

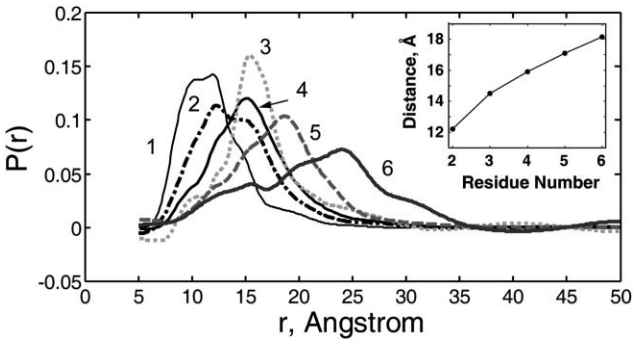

Fig. 3 Distance distributions obtained from DQC data in Fig. 2 for double-labeled peptoids **1–6** as calculated from inverse reconstruction by Tikhonov regularization. Maxima shift to longer distances as the residue spacing between labeled sidechains increases. Note that **6**, lacking branched structure-inducing sidechains, exhibits a wide distance distribution. Inset shows mean distances as a function of inter-residue spacing for **1–5**.

presence of the TEMPO spin probes does not substantially compromise the formation of stable helical secondary structure by peptoids incorporating branched chiral sidechains. Inter-probe distance measurements and corresponding variances were obtained from analysis of DQC-ESR data of double-labeled peptoids. Distances were observed to increase as a function of the number of monomer units separating the two nitroxide radicals. The tendency for bulky chiral sidechains to establish conformational ordering in the peptoid backbone was evident in the narrow distance distributions of oligomers incorporating such sidechains. We anticipate that the TEMPO group will be highly useful as a probe of structure and dynamics in peptoids and other folded oligomers. The use of pulsed ESR spectroscopy may be an effective means to evaluate the presence of compact folded species in macromolecular peptoid constructs.[3] Furthermore, generating a set of distance measurements as constraints in triangulation[5a,c,15] algorithms may enable the definition of peptoid folding topologies.

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Notes and references

‡ For peptoid **7**, liquid chromatography–mass spectrometry (LC–MS) performed on the crude cleavage products and on the predominant product isolated by semi-preparative HPLC identified a compound with a mass one Da higher than the desired product. For peptoids **1–6** incorporating two nitroxides in the sequence, a mass two Da higher than expected was observed. These observations are consistent with results obtained elsewhere on the TFA cleavage of TEMPO containing peptides, in which the increased masses were attributed to the formation of the corresponding hydroxylamines.[10,16] In agreement with these studies, we observed that the TFA cleavage products of **1–7** were reverted back to the free radical form nearly quantitatively upon treatment with aqueous ammonia. After aqueous ammonia treatment, analysis of the crude cleavage products showed only one major peak by HPLC at substantially increased retention times, with masses corresponding to the desired nitroxides (see ESI†).

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