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Extreme Stability of Helices Formed by Water-Soluble Poly-*N*-Substituted Glycines (Polypeptoids) with α-Chiral Side Chains

Abstract: *Poly-N-substituted glycines or "peptoids" are protease-stable peptide mimics. Although the peptoid backbone is achiral and lacks hydrogen-bond donors, substitution with α-chiral side chains can drive the formation of stable helices that give rise to intense CD spectra. To systematically study the solution properties and stability of water-soluble peptoid helices with α-chiral side chains, we have synthesized and characterized an amphipathic, 36-residue N-substituted glycine oligomer. CD was used to investigate effects of concentration and solvent environment on this helical peptoid. We saw no significant dependence of helical structure on concentration. Intense, "α-helix-like" CD spectra were observed for the 36-mer in aqueous, 2,2,2-trifluorethanol (TFE), and methanol solution, proving a relative insensitivity of peptoid helical structure to solvent environment. While CD spectra taken in these different solvents were fundamentally similar in shape, we did observe some interesting differences in the intensities of particular CD bands in the various solvents. For example, the addition of TFE to an aqueous solvent increases the degree of peptoid helicity, as is observed for polypeptide α-helices. Moreover, the helical structure of peptoids appears to be virtually unaffected by heat, even in an aqueous buffer containing 8* M *urea. The extraordinary resistance of these peptoid helices to denaturation is consistent with a dominant role of steric forces in their structural stabilization. The structured polypeptoids studied here may have potential as robust mimics of helical polypeptides of therapeutic interest.* © 2002 John Wiley & Sons, Inc. Biopolymers 63: 12–20, 2002

Keywords: *poly-N-substituted glycines; peptoids; protease-stable peptide mimics; peptoid backbone; chirality; oligomer*

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Table I ***N*-Substituted Glycine Side Chains**

H–(N(R)–CH₂–C(=O))$_n$–NH$_2$	*N*-Substituted Glycine Oligomer or Polypeptoid
R = side chain[a]	Designator
H, CH_3, phenyl	*N*spe = (S)-*N*-(1-phenylethyl)glycine
H, CH_3, C(=O)OH	*N*sce = (S)-*N*-(1-carboxyethyl)glycine

[a] These structures refer to the *N*-substituent of a glycine monomer.

INTRODUCTION

Helical secondary structure in folded proteins and polypeptides may be stabilized by a number of different forces, including hydrogen bonds, van der Waals forces, hydrophobic interactions, and electrostatics.[1–4] Significant disruption of one or more of these stabilizing forces by environmental factors will typically result in cooperative denaturation of folded structure.[5,6] The propensity of protein secondary and tertiary structures to unravel and aggregate in adverse environmental conditions is generally a nonideal molecular property for their therapeutic and biomaterial applications.[7] The protease susceptibility of natural proteins is another factor that limits their bioavailability as medicines and utility as biomaterials.

Recently, progress has been made towards the development of several new classes of peptidomimetic oligomers, for example β-peptides,[8–13] γ-peptides,[14,15] poly-*N*-substituted glycines (peptoids),[16–19] peptide nucleic acids,[20] oligoureas,[21] sialooligomers,[22] and aedemers.[23] These novel oligomer systems are designed to capture some of the self-organizing features of biopolymers, where specific sequence and chain length enable the adoption of a defined "fold," while offering greater chemical diversity, stability to enzymatic degradation, and more robust secondary structure.[24–26] Depending upon their specific chemical structures and monomer sequences, members of these oligomer families are soluble and exhibit stable secondary structure in a variety of different organic and aqueous solvents.

The development of non-natural oligomers with biomimetic folded structure that remains relatively impervious to environmental perturbations will facilitate the design of robust biomaterials with a long shelf life and a wide therapeutic window. One interesting approach to engineering such structural stability in biomimetic oligomers is to design molecules for which strong steric (van der Waals) forces are the predominant influence in secondary structure formation. Whereas natural protein secondary structure, which is stabilized by a complex hierarchy of different forces, is highly sensitive to environmental conditions, secondary structure directed by mutual steric avoidance of bulky groups is expected to be more robust.

The present study focuses on the response of polypeptoid-based secondary structure to variations in their solvent and thermal environment. Peptoids are sequence-specific oligomers based on a polyglycine backbone, with side chains appended to the tertiary amide nitrogen (Table I).[16] As they lack amide protons, peptoid oligomers cannot form backbone–backbone hydrogen bonds such as those that stabilize peptide α-helices. Moreover, since the *N*-substituted glycine backbone lacks chiral centers, peptoid chains composed of predominantly achiral side chains have no propensity to adopt stable secondary structure (i.e., a peptoid with achiral side chains may form a helix; however, this helix will rapidly interconvert between both screw senses without preferring either).[18] However, substitution of oligopeptoids with at least one-half α-chiral, aromatic side chains such as the (S)-*N*-

Table II Peptoid Oligomer Characteristics

Peptoid	Monomer Sequence	Molar Mass Calculated:Found	% Full-Length Oligomer in Crude Prep[a,b]
1	$(N\text{sce}N\text{sce}N\text{spe})_{12}$	5050.2:5050.7	16

[a] As determined by analytical reverse phase (RP-HPLC) of crude product.
[b] Crude material was purified by preparative RP-HPLC to ~ 90% chain length homogeneity before analysis by CD.

1-phenylethyl group (*N*spe, Table I) or the corresponding (R) enantiomer *N*rpe has been shown to direct the formation of chiral, helical structure through imposed steric constraint.[17–19] This effect of side-chain chirality on an achiral polymer backbone was previously observed for helical polyisocyanates.[27]

Here, we take advantage of the strong and distinct CD signature of these chiral peptoid helices[17,18] to systematically investigate the effects of solvent polarity, addition of denaturant, and elevated temperature on conformational stability. We find that the helical structure of a peptoid 36-mer composed of one-third *N*spe and two-thirds (S)-*N*-1-carboxyethyl (*N*sce) side chains is extremely robust in both aqueous and polar organic solution.

MATERIALS AND METHODS

Amine Submonomers for Peptoid Synthesis

The (S)-*N*-(1-phenylethyl)glycine (*N*spe) monomer was prepared from the amine (S)-1-phenylethylamine, purchased from the Aldrich Chemical Co. (Milwaukee, WI) at 99.5% purity. (S)-*N*-(1-carboxyethyl)glycine (*N*sce) was prepared from (L)-*O*-*t*-butyl-alanine-HCl (Novabiochem; San Diego, CA).[17] Table I shows the structures of the peptoid monomers derived from these amines.

Peptoid Synthesis and Purification

Solvents and reagents were purchased from commercial sources and used without further purification. A peptoid 36 residues in length with the sequence $(N\text{sce}N\text{sce}N\text{spe})_{12}$ (Table II) was synthesized on a 433A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) with in-house software modification to the optimized submonomer protocol of Zuckermann et al.,[16,17] and was analyzed and purified by reversed-phase high performance liquid chromatography (HPLC) as described.[16–18] Electrospray ionization mass spectrometry (ESI-MS) of the purified synthesis product, to confirm molecular weight and purity, was performed by Northwestern University's Analytical Services Laboratory (Table II). CD studies were performed as described.[18] Peptoid oligomer stock solutions (~ 0.4 m*M* in acetonitrile/aqueous (1:1) solution, with the concentration accurately known) were used for preparation of CD samples, after titration with 5 *M* sodium hydroxide solution to produce anionic peptoids that were fully deprotonated for maximal water solubility.[5] The stock solution was diluted in the solvent of interest, to a final concentration of ~ 20 μM (with the concentration accurately known) for use in CD studies. All CD spectra reported here represent the average of 40 accumulations of data over the wavelength range of 178–280 nm.

RESULTS AND DISCUSSION

Synthesis and Purification of a Sequence-Specific, Water-Soluble Peptoid Oligomer

An oligopeptoid composed of 36 residues with the sequence $(N\text{sce}N\text{sce}N\text{spe})_{12}$ was synthesized. Crude oligomer purity with respect to the concentration of the full-length 36-mer (i.e., the degree of monodispersity) was determined by reversed-phase HPLC (Table II). Compound **1** was purified using preparative HPLC to ~ 90% homogeneity before further study. Molar mass determined by ESI-MS was in agreement with the expected value (Table II).

Effects of Concentration on Oligopeptoid Secondary Structure

We first confirmed that the shape and intensity of the CD spectrum of **1** is completely independent of peptoid concentration, by taking CD spectra at several concentrations between 1 μM and 104 μm in 5 m*M* sodium phosphate buffer, pH 7.0 (data not shown). When the data are plotted as per-residue molar ellipticity, all of these spectra overlap, indicating an absence of peptoid helix structural stabilization by intermolecular association over the concentration range studied. A characteristic CD spectrum, reminiscent in

shape and intensity of that for polypeptide α-helices, has been observed for a variety of peptoid oligomers that are comprised of at least one-third α-chiral, aromatic side chains.[17–19] Yet it has been established by two-dimensional (2D)-NMR structural studies in methanol solution[28] that oligopeptoids exhibiting this distinct CD spectrum adopt right-handed helices with *cis*-amide bonds, approximately 3 residues per turn, and a pitch of ~ 6 Å. Hence, the secondary structure of this class of polypeptoids is more similar to that of a polyproline type I helix than to a peptide α-helix, which has *trans*-amide bonds in the backbone.

Salt and Solvent Effects on Oligopeptoid Secondary Structure

Compound **1** is soluble in water as well as in mixed 2,2,2-trifluoroethanol (TFE)/water and pure methanol. We have systematically studied the effects of each of these solvent environments on the stability of polypeptoid secondary structure as reported by CD. To prepare the samples for these studies, a 0.4 m*M* stock solution of the trifluoroacetic acid (TFA) salt of compound **1** (with volume ~1 mL) was titrated with 3 μL of 5 *M* NaOH to ensure that *N*sce side chains (pK_a ~ 4) were predominantly in the ionized state. This procedure produced the 24-valent sodium salt of **1,** which was then diluted into various buffers and solvents to a final concentration of ~ 20 μ*M.*

First, we investigated the effects of ionic strength on the helical structure of **1** in aqueous solution at room temperature. CD spectra taken in pure, deionized water (unbuffered, pH 6.0) as well as in 5.0 m*M,* 150 m*M,* and 1 *M* sodium phosphate buffers (all, pH 7.0) are shown in Figure 1. We find that at low sodium phosphate concentrations (up to 5.0 m*M*), ionic strength has no apparent effect on the degree of helicity of peptoid **1,** as shown by the identical shape and intensity of spectra acquired in deionized water and in 5.0 m*M* salt. Hence, even when the strength of charge–charge repulsive forces between ionized *N*sce side chain moieties is expected to be great, a stable peptoid helix is formed. This is in contrast to the behavior of strongly ionic, α-helical peptides, which are often unstructured in the absence of screening by counterions.[29] This difference in the behavior of ionic peptoid **1** and typical ionic peptides may be a consequence of the looser helical pitch of these peptoids (~ 6 Å),[28,30] which produces a helix with 3 residues/turn and ~ 120° between neighboring monomers. Peptide α-helices, on the other hand, have a pitch of 5.4 Å, 3.6 residues per turn, and a separation between residues of ~ 100° around the helical axis, hence allowing for greater interaction.[5] On the other hand,

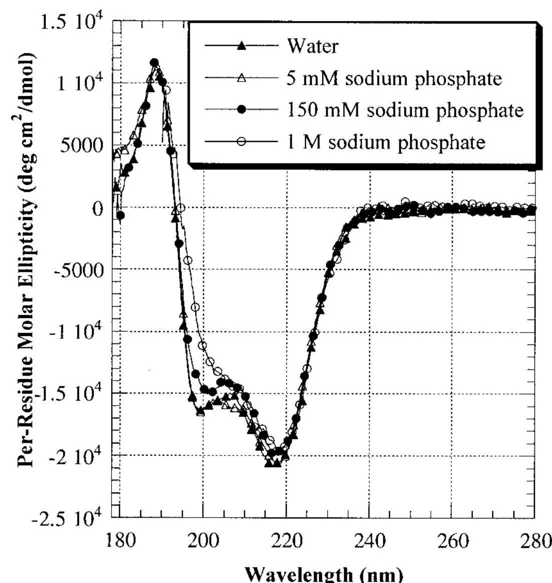

FIGURE 1 CD spectra of **1** (*N*sce*N*sce*N*spe)$_{12}$ at ~20 μ*M* in varying concentrations of sodium phosphate from 0.0 m*M* (i.e., pure deionized water) to 1 *M.*

with 3 residues per turn, interactions between peptoid side chains that are "stacked" in the type of helix studied here would be expected to significantly influence their structure and stability. We have previously observed this effect in a study of oligopeptoids comprised of mixed chiral, achiral, aromatic, hydrophobic, and polar side chains.[19]

Spectra acquired in the higher salt conditions of 150 m*M* (Figure 1) and 75 m*M* sodium phosphate (data not shown) also overlap with each other in shape and intensity, but show a small reduction in magnitude and a 3-nm red shifting of the minimum at 200 nm. In the high salt condition of 1 *M* sodium phosphate, when charge–charge repulsion between *N*sce side chains should be extensively screened, a small but notable alteration of the spectrum is observed. In particular, the peak at ~ 200 nm is reduced in magnitude and is red shifted by ~ 5–6 nm in comparison to that in pure water, beginning to resemble a shoulder on the major CD peak at 218 nm. There is essentially no change in the spectral intensity of the bands of ellipticity at 188 nm and 218 nm. In light of the discussion above about helical pitch, one interpretation of this data is that charge–charge repulsion between *N*sce side chains in lower ionic strength solutions does, in fact, subtly alter the most stable helical configuration of this family of polypeptoids. Indeed, it has recently been recognized that in protein α-helices, local distortions in structure from a canonical α-helix configuration are much more common than previously recognized.[3] Hence, some distortion of the expected,

polyproline-like peptoid helix[28,30] for **1** in the absence of charge screening seems reasonable, especially in light of the fact that in comparison to spectra taken in pure water, the spectrum of **1** in 1*M* salt (i.e., with strong counterion screening) more closely resembles the prototypical CD spectra we have observed in previous studies of long (> 13-mer), fully helical peptoid homooligomers of (R)- or (S)-*N*-(1-phenylethyl)glycine in acetonitrile solution (Ref. 18, and data not shown).

These relatively subtle effects aside, we see that the helical structure of peptoid **1,** which is composed of two-thirds anionic side chains, is only mildly changed by the degree of electrostatic screening provided by counterions. The lack of a dramatic salt effect for these anionic polypeptoid helices is interesting to contrast with observations of charged polypeptide helices in solutions of different ionic strengths. Addition of salt has been shown to stabilize secondary structure in a variety of ionic peptide sequences in water, through masking of the electrostatic repulsion between side chains.[31–34] Peptide helices that are formed by sequences with a high fraction of charged side chains such as lysine are completely destabilized in low ionic strength conditions.[29] In general, effects of salt and solvent screening on the helicity of peptide sequences that include both charged and hydrophobic side chains tend to be complex.[35–37] Perhaps since the polypeptoid helices studied here are more "loosely wound" than peptide α-helices, charge–charge repulsion between ionic side chains produces more subtle, less "catastrophic" effects on helical structure.

Addition of TFE to an aqueous solvent at 0–20 mol % has been shown to stabilize helical structure in polypeptides.[38–40] Other aliphatic alcohols, such as methanol, can have a similar though weaker effect.[5,41,42] Increase in polypeptide helicity with TFE addition has been attributed to TFE-mediated loosening of the aqueous solvent shell around helical conformations[39,40] and to preferential interaction of TFE molecules with hydrophobic side chains[40] and peptide carbonyl groups.[43] In alcohol solution, the solvation free energy of peptides varies less strongly with conformational fluctuations than in aqueous solutions.[44] Thus, the overall stability of secondary structure in alcohol is governed primarily by the conformational energy of the polypeptide backbone, as dictated by the preferred ϕ–Ψ bond angles[44] and less by particulars of side-chain sequence. This allows a polypeptide to more easily develop its lowest-energy conformation in an alcohol solution, *via* steric rearrangements as well as by intramolecular hydrogen bonding (i.e., formation of the classical α-helix).[44] To further investigate the extent of analogy between peptoid and peptide helices, we have examined the effect of TFE and methanol on the structure of a water-soluble polypeptoid helix.

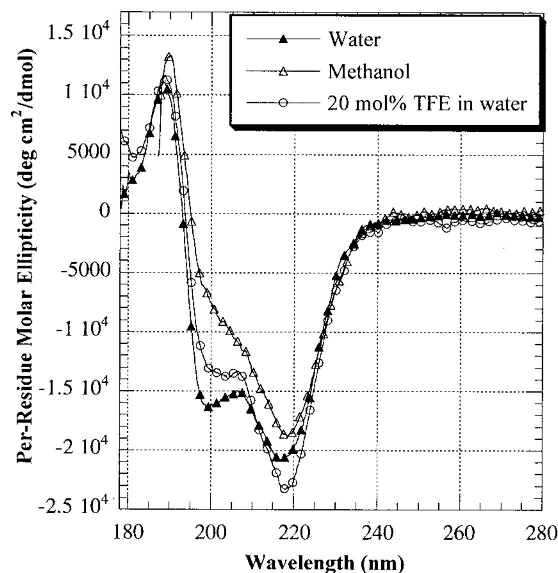

FIGURE 2 CD spectra of **1** at ~20 μM in three different solvents (water, methanol, and 20 mol % TFE in water), taken at room temperature.

We find that the CD spectra of **1** in 20 mol % TFE/water and methanol solutions are fundamentally similar to those observed in aqueous solutions (Figure 2). That is, the spectra are intense and show two minima at ~ 200 and 218 nm and a maximum at 188 nm. However, CD spectra acquired in these solvents do display a few subtle but notable differences from that obtained in water. In mixed TFE/water, we observe that the CD minimum at 200 nm is reduced to a shoulder, while the band at 218 nm is concomitantly *increased* in intensity. The 188 nm maximum remains identical to that in water. The band of ellipticity at 218 nm is often considered most representative of the degree of helicity in polypeptides.[45] We conclude that, analogously, the observed strengthening of this CD minimum with addition of TFE indicates increased helicity of peptoid oligomer **1** in the presence of this highly polar alcohol. It is interesting that this ordering effect is also observed with polypeptoid helices, although they are stabilized by a wholly different balance of forces than polypeptide α-helices.[3,38,39] Intriguingly, the TFE-influenced spectrum is similar to that observed in high-ionic strength aqueous solutions, i.e., more similar in shape to the fully developed CD spectrum of a long, organosoluble *N*spe polypeptoid[18] than is the spectrum taken in pure water. Hydrophobic interactions of TFE molecules with *N*spe

side chains[40] or TFE hydrogen bonding with carbonyls in the backbone or side chains[43] might allow the peptoid to adopt its lowest-free energy configuration, by alleviating helix distortions resulting from poor solvation of stacked *N*spe side chains or from ionic repulsion between charged *N*sce side chains. The essential features of the CD spectrum of **1** in methanol are again quite similar to that observed in a 1 *M* sodium phosphate solution or in TFE/water, however we see an overall, 20–30% reduction in the intensity of CD (Figure 2).

As peptoids are derivatives of a polyglycine backbone and adopt solution conformations similar to those of polyproline type I helices,[28,30] it is interesting to recall and consider the effects of dissolution in various solvents on polyprolines. Polyproline oligomers can exist in two conformational states, called forms I and II. Form I is a right-handed helix with all peptide bonds in the *cis* configuration, and is stable in pyridine and aliphatic alcohols. Form II is a left-handed helix with all peptide bonds in the *trans* configuration, and is stable in water, acetic acid, formic acid, and benzyl alcohol.[46–48] Generally speaking, the ratio of *cis/trans* peptide bonds in proline residues will increase with increasing solvent polarity [i.e., $CCl_4 < CDCl_3 < (CD_3)_2CO < D_2O$].[49] In aqueous solution, where form II helices will predominate, more complete conversion of residual form I to form II helices can be driven by the addition of urea.[50] In comparison to the extreme sensitivity of polyproline helix backbone configuration to solvent polarity, poly-*N*-substituted glycine helices longer than 12 residues stably retain a *cis*-amide configuration. Hence, polypeptoid helices can be considered configurationally more stable than the polyprolines, and much less sensitive to their solvent environment.

The Effect of Urea on Oligopeptoid Secondary Structure

Urea is a chaotrope that effectively stabilizes the unfolded forms of peptide α-helices and other types of secondary structure by breaking water's hydrogen-bond network, interacting with peptide groups, and reducing the solvophobic driving force for chain folding and collapse.[51] Single, isolated peptide helices also denature cooperatively in urea solutions.[52] We performed CD analysis of **1** at room temperature, dissolved in 5 m*M* sodium phosphate buffer (pH 7.0) with varying concentrations of urea ranging from 2 to 8 *M* (Figure 3). Due to the absorbance of urea in the buffer, only the 280–205 nm range was available for investigation. As seen in Figure 3, we find that the CD band at 218 nm (most reflective of the degree of helicity[45]) shows essentially no change in intensity with increased urea concentration. Thus, it appears that the helical structure of peptoid **1** remains stable in 5 m*M* sodium phosphate buffer even at urea concentrations of up to 8 *M*. Hydrogen-bonding of urea with peptide bonds has been proposed to be a major driving force for the urea denaturation of polypeptide α-helices.[52] As peptoids lack amide protons, the polypeptoid backbone has fewer modes of specific interaction with urea molecules than does a polypeptide. Interactions of urea with the aromatic and hydrophobic side chains (*N*spe), which make up one-third of the peptoid sequence studied here, are apparently not a strong force for peptoid helix denaturation, although these interactions have been claimed to be important in protein denaturation.[53]

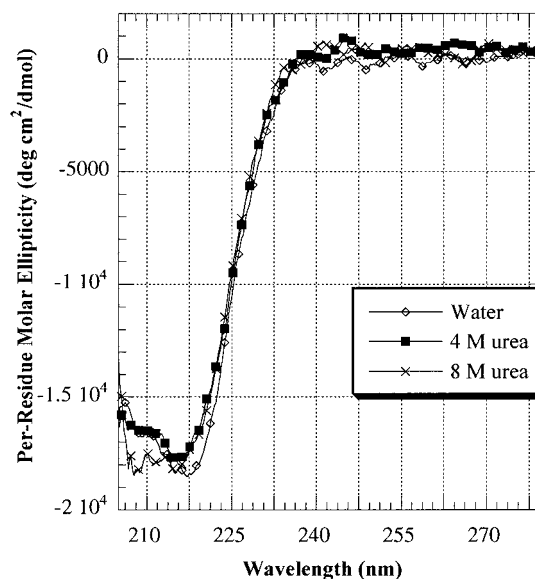

FIGURE 3 CD spectra of **1** at ~20 μ*M* in different concentrations of urea in a 5 m*M* sodium phosphate buffer. The CD spectrum of **1** in water is included for reference.

The Effect of Temperature on Polypeptoid Secondary Structure

We were interested in whether peptoid helical structure could be forced to denature under more severe conditions. As a point of comparison, it is interesting to consider what has been observed for polyproline helices upon heating. While an increase in temperature to 55°C was found to enhance chain motion and flexibility in polyproline helices, and to cause a linear reduction of magnitude in the bands in the CD spectrum, cooperative unfolding was not observed.[54,55] In prior work, temperature-dependent CD studies have been performed on two different water-soluble pep-

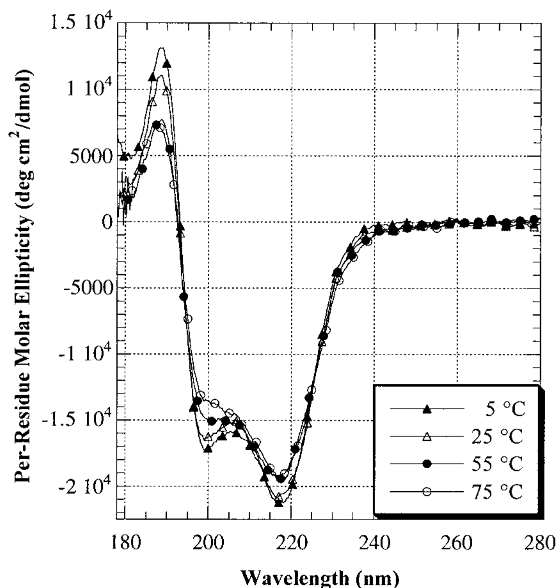

FIGURE 4 A CD temperature study of compound **1** dissolved in water (~20 μM), with observation of a minimal amount of thermal disordering. Two isodichroic points are evident.

toid hetero-oligomers; helical structure was found to be stable up to 65°C.[17] Similarly, organosoluble peptoid homo-oligomer up to 20 monomers in length exhibit only minimal disordering in acetonitrile solution at temperatures up to 75°C.[18] Several other biomimetic oligomers systems, including β-peptides[9,13] and γ-peptides[14] also have been found to form more stable helical structures than α-peptide helices.

To probe the stability of peptoid helices in a systematic manner, we obtained CD spectra of polypeptoid **1** at 10°C increments between 5 and 75°C in water (Figure 4), 5 m*M* sodium phosphate buffer (data not shown), and methanol solution (data not shown). Temperature studies in the three different solvents showed similar results. The intensity of the spectra are slightly and monotonically reduced by heating in these solvents, and spectra pass through isodichroic points at ~ 227 and ~ 193 nm (as seen in Figure 4 for water) in all three solvent systems. In polypeptides, the presence of similar isodichroic points is generally taken to indicate the existence of a two-state transition, from one predominant conformational state to another.[56] The overall shapes of the peptoid CD spectra in water remain relatively stable from 5 to 75°C, except for the CD minimum at ~ 200 nm, which is decreased in magnitude and begins to resemble a shoulder at 75°C (as was observed in 1 *M* salt and TFE solutions). This provides evidence that only minor structural changes to the helix occur with increased temperature. Similarly, β-peptide secondary structures, which are stabilized by strong steric interactions as well as by hydrogen bonding, exhibit substantial thermodynamic stability in methanol solution, with only minimal changes in their NMR and CD spectra upon heating.[13]

Finally, a similar CD melting study was performed with **1** dissolved in sodium phosphate buffer in the presence of 8 *M* urea (Figure 5). Strikingly, even in 8 *M* urea, the CD band at 218 nm shows only minimal reduction in intensity upon heating from 25 to 75°C; its original magnitude is regained at 25°C. Thus, peptoid **1** apparently retains a stable helical configuration in an 8 *M* urea, 5 m*M* sodium phosphate buffer, even at temperatures of up to 75°C.

These temperature and solvent studies provide a useful comparison of the stability of oligopeptoid helices to polypeptide α-helices. It is common to denature protein secondary structure with the addition of relatively small amounts of either heat or chemical denaturant.[5] In the present study, neither high temperature nor 8 *M* urea (alone or in combination) succeeded in denaturing the helical secondary structure of peptoid oligomer **1.** Hence, this work has demonstrated the extraordinary conformational stability of the secondary structure of this water-soluble polypeptoid, which is stabilized primarily by avoidance of steric clash. This class of peptoid oligomers appears, therefore, to form highly thermostable helices under any condition in which the molecules themselves are soluble. These results suggest that poly-*N*-substituted glycines are a promising class of oligomers with

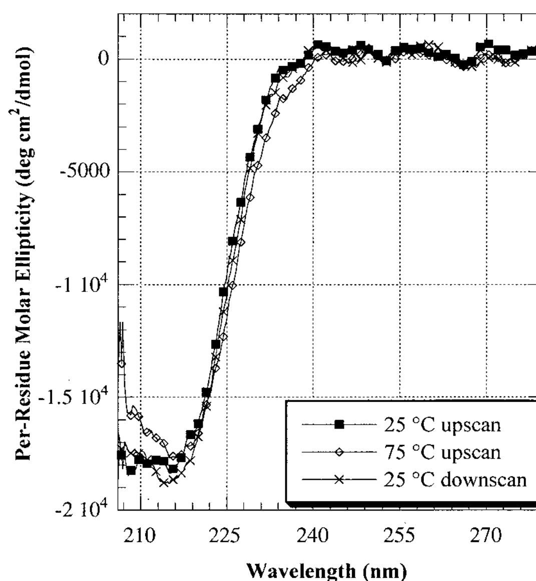

FIGURE 5 A CD melting study of **1** at ~20 μM dissolved in 8 *M* urea, 5 m*M* sodium phosphate buffer. Both temperature upscan and downscan are shown.

which to develop robust biomaterials and therapeutics.

CONCLUSIONS

We have performed a careful and systematic examination of the helicity of the polypeptoid (*N*sce*N*sce*N*spe)$_{12}$ as a function of peptoid concentration, solvent, and temperature. We find no effect of concentration on helicity. In detailed studies of this polypeptoid, we have found that unlike polypeptide α-helices, environmental conditions have very little influence on secondary structure. Ionic strength has only subtle effects on structure in sodium phosphate solutions, up to concentrations of 1 *M* salt. CD spectra display only small changes in shape and intensity as a function of solvent polarity, in water, water/TFE, and methanol solution. Interestingly, we find that as for polypeptide α-helices, the addition of TFE to an aqueous solution of polypeptoids increases their degree of helicity. Moreover, the extent of resistance of peptoid helical structure to thermal disordering, even in an 8 *M* urea solution, is extraordinary. Since peptoid secondary structure is highly insensitive to solvent environment and thermal perturbations, this study provides confirmation that this type of helical structure must be stabilized primarily by steric factors. Understanding how solvent and temperature affect the helical structure of this class of polypeptoids enhances our ability to design these molecules for broad applications requiring solvation in different environments, especially for settings that require structural stability during environmental changes. We are working to exploit the amphipathic nature of similar polypeptoid helices to create ultrastable mimics of polypeptides that interact with lipid bilayers.

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