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COMMUNICATION

# Conformational control via sequence for a heteropeptoid in water: coupled NMR and Rosetta modelling

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**We report a critical advance in the generation and characterization of peptoid hetero-oligomers. A library of sub-monomers with amine and carboxylate side-chains are combined in different sequences using microwave-assisted synthesis. Their sequence-structure propensity is confirmed by circular dichroism, and conformer subtypes are enumerated by NMR. Biasing the $\psi$-angle backbone to *trans* (180°) in Monte Carlo modelling favors *i* to *i+3* naphthyl-naphthyl stacking, and matches experimental ensemble distributions. Taken together, high-yield synthesis of heterooligomers and NMR with structure prediction enables rapid determination of sequences that induce secondary structural propensities for predictive design of hydrophilic peptidomimetic foldamers and their future libraries.**

Peptoids are peptidomimetic oligomers composed of *N*-substituted glycine units with potential applications in fields spanning the biomedical to the materials sciences, provided their macromolecular structures can be controlled.[1-8] Peptoids have the same C-C-N backbone repeat as naturally-occurring proteins, but the N-substitution makes the backbone achiral and precludes any (protein-like) backbone hydrogen-bonding networks (Fig. 1).[9,10] Chirality and backbone hydrogen-bonding are responsible for energetically-preferred local structural biases in proteins and are key elements of 'secondary structure'. These protein secondary structures, in combination with side-chain interactions and chemical functionality (i.e. hydrophobicity, polarity, π-stacking, and hydrogen bonds), dictate three-dimensional folding and function. Thus, the challenge for peptoid-based foldamers, is developing control over structure and design at the level of proteins. This challenge requires identification of sequence-to-structure relationships, while developing comprehensive methods to generate, *individual, self-folding, water-soluble* hetero-peptoids.[11-13] The first step toward this goal is to design and model peptoids with the chemical and structural functionality of protein secondary structures.

Encouragingly, several publications suggest individual peptoids can achieve three-dimensional order. Wu et. al. and Patch et. al. demonstrated that hydrophobic peptoid homooligomers, could assume secondary structure in organic solvents or when embedded into lipid membranes.[14,15] Armand et. al. demonstrated that three-dimensional order can be achieved for a hetero-oligomeric pentamer in methanol.[16] Finally, Darapaneni et. al. partially improved upon hydrophobic peptoid solubility by post-synthetic addition of piperazines to a homooligomer, demonstrating that the peptoid maintained its secondary structure once transferred into aqueous phase.[17] Most of these studies used bulky chiral side-chains to reduce peptoid conformational freedom, providing an approach to generate secondary structural elements.[14-18] Unfortunately, these peptoids lack the structural and sidechain chemical precision of proteins, or their hydrophilicity, precluding downstream complex functions such as catalysis and molecular recognition in any peptoid foldamer. Further, many of these studies only rely on circular dichroism (CD) to

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characterize structures.[18-23] Finally, beyond the limited structural understanding achievable from ensemble measurements (e.g. CD cannot resolve ensemble heterogeneity as well as NMR), past modelling of peptoid structures have assumed that peptoid backbones had a flexibility similar to peptide backbones: limiting the potential for studying the driving forces for peptoid secondary structure formation.

These facts open many opportunities for greatly improving aqueous peptoid design by merging the insights obtained from previous analyses of peptoids[24-27] and leveraging refined synthetic protocols with characterization by nuclear magnetic resonance spectroscopy (NMR)[17] and molecular modelling.[28-34]

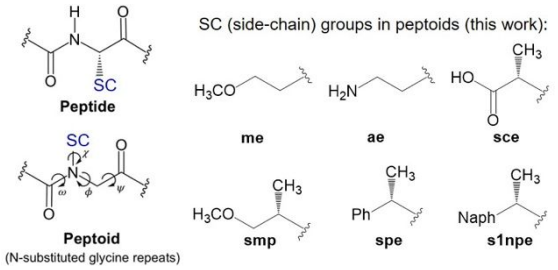


**Fig. 1.** Structural comparison of peptide and peptoid backbones (backbone dihedral angles ω, φ, ψ, and side-chain angle $\chi$ shown). SC represents the side-chain group.

In this study we demonstrate an integrated methodology for developing short hetero-oligomers of individual, water-soluble peptoids, with conformational biases based on sequence. Our aim is to identify patterns that limit the secondary structure palette sufficiently to allow folding to occur, and to measure the degree of diversity – conformational freedom – in induced secondary structures. Using a microwave synthesis method that facilitates efficient hetero-peptoid synthesis, and incorporates polar, hydrophilic side-chains with amine and carboxyl functional groups, we achieved high-purity syntheses. We refined the ensemble characterization of CD by incorporation of NMR to determine the diversity of structures in solution. Finally, we employed a modelling technique with demonstrated success for peptides, and extensible methods for peptoids[34-36], and for the first time demonstrated the importance of enforcing peptoid-specific backbone parameters (i.e. $\psi \sim 180^{o}$).[28,29] The success of this approach is highlighted for a pentameric peptoid sequence that exhibits a restricted set of conformers in aqueous solution.

The submonomer solid-phase synthesis developed by Zuckermann is ubiquitous for synthesis of up to 50-mer peptoids (Supporting Information, Scheme S1, ESI†).[37,38] However, long reaction times (>2.5 hrs per residue), high reagent stoichiometry requirements, and lack of commercial precursors for many side-chains limit the potential for high-throughput combinatorial production of peptoids.[39]

Microwave irradiation can accelerate solid-phase synthesis of peptoids; however, previous applications focused exclusively on homo-oligomeric non-water soluble peptoids and used excess submonomer precursors.[40,41] Here we expand this technique to the domain of water-soluble peptoid heteropolymers, enabling an economical synthetic method that is amenable to diverse submonomer precursors.

The various submonomers used to construct our peptoid hetero-oligomer library are shown in Fig. 1. Critically, these side-chains supply hydrophobicity, hydrophilicity, chirality, and hydrogen bonding otherwise absent from peptoid backbones. Further, we incorporated chiral phenyl (*N*spe) or chiral naphthyl (*N*s1npe) submonomers periodically at *i* and *i+3* positions in order to favor π-π stacking, which we anticipated could induce polyproline type-I-like turns.[42,43]

To compare the performance of microwave-assisted synthesis to the conventional batch submonomer method, we first synthesized an α-chiral pentameric peptoid with naphthyl (Ns1npe) groups (**H5** in Table 1).[14] We found that conventional synthesis of **H5** at room temperature required approximately 21 hrs for coupling five peptoid submonomers. This reaction required high stoichiometric equivalents of bromoacetic acid, N,N'-diisopropylcarbodiimide (DIC) and submonomers to produce crude **H5** at 66% purity. Under optimized microwave conditions the reaction time was reduced to approximately 2.5 hrs, required fewer equivalents of submonomers, and produced crude **H5** at 90% purity (Supporting Information Fig. S1, ESI†). Additional purification to >95% homogeneity was achieved via semi-preparative reverse-phase HPLC (Fig. S2, for details see ESI†), and these samples were used in our structural analyses.

While purity in any one synthesis can be achieved by post-synthetic purification, the crude purity of our techniques will be a critical feature of future, high-throughput combinatorial applications and peptoid design.

**Table 1.** Microwave synthesis of peptoid hetero-oligomers

| entry | peptoid sequence[a] | MS[b] | purity (%)[c] |
|---|---|---|---|
| **H1** | H-Nspe-Nsce-Nme-Nspe-Nsce-$NH_2$ | 713.5 | 94 |
| **H2** | H-Nspe-Nae-Nme-Nspe-Nae-$NH_2$ | 655.4 | 91 |
| **H3** | H-Nae-Nspe-Nae-Nme-Nspe-$NH_2$ | 669.8 | 90 |
| **H4** | H-Ns1npe-Nae-Nme-Ns1npe-Nae-$NH_2$ | 754.9 | 87 |
| **H5** | H-Ns1npe-Nae-Nae-Ns1npe-Nsce-$NH_2$ | 768.9 | 90 |
| **H6** | H-Nae-Nspe-Nme-Nae-Nspe-$NH_2$ | 654.8 | 84 |
| **H7** | H-Nspe-Nae-Nsmp-Nspe-Nae-$NH_2$ | 668.8 | 87 |

[a] Peptoids were synthesized on Rink amide resin (0.05 mmol scale). Nae submonomer as Boc-protected and Nsce submonomer as *tert*-butyl protected. [b]ESI-MS data. [c]Percent purity estimated by analytical reversed-phase HPLC of crude dry peptoid after ether precipitation and washing.

To demonstrate sequence effects on the structure of peptoids, a small library of hetero-oligomers, with an expanded repertoire of chemical functionality, was prepared by our microwave protocol on a solid support (Table 1). Collectively, good to excellent (84-94%) crude purity was obtained in short (5-hr) reactions for all sequences in Table 1. Moreover, we were able to synthesize octamer hetero-oligomers (**H8** and **H9**) with moderate (62-68%) purities (Table S1).

Secondary structure preference was first assessed using CD. Each peptoid showed distinct CD patterns, varying in peak wavelength and intensity (degree of ellipticity), suggesting the CD pattern depends on the sequence (Fig. S3, ESI†). In contrast to phenyl-containing peptoids (H1, H2, H3, H6 and H7, Supporting Information Fig. S3A,

 

ESI†), the naphthyl-containing peptoids (**H4** and **H5**, Supporting Information Fig. S3B, ESI†) show strong features at 230 nm and 215 nm, suggesting a chiral preference induced in the backbone structure. While **H4** and **H5** have the same number of naphthyl groups, the **H4** peaks are larger and sharper, suggesting a more stable structure than **H5** (Fig. S3B, ESI†), as also observed in NMR. Subsequent analyses therefore focus strictly on structures of **H4**.

Assignments by Fuller *et. al.* suggests that minima near 230 nm are analogous to helix bands in peptides, and represent *i* to *i+3* interactions consistent with naphthyl-naphthyl stacking.[44] However, the lack of defined aqueous solution phase structures of hetero-peptoids limits the interpretation of CD for these compounds and only describes their average helicity.[16]

We employed NMR to determine the occupancies of distinguishable conformational motifs. This contrasts with how NMR is normally employed, where peaks are assigned, coupling provides connect information, and through-space coupling provides distance information leading to determination of a structure. However, in complex mixtures it is nearly always the case that NMR peaks can be observed but not assigned. Even so, critical information can often be found in the spectra. When conformational motifs comprise the majority of signal, we can partition these into states and determine an occupancy ratio. Thus, we can estimate the occupancy of certain conformers even though we can neither determine structures nor assuredly associate NMR peaks with particular defined states.

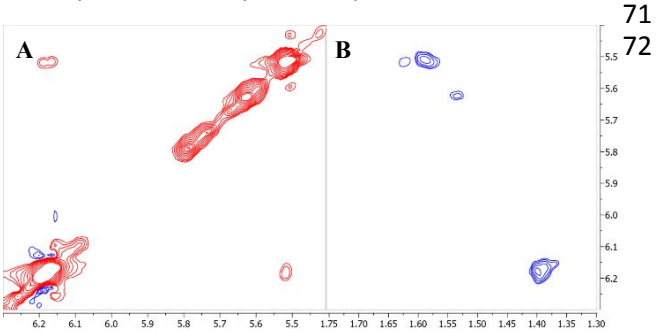


**Fig. 2.** Structural analysis of **H4** using ROESY spectra (400 ms mixing time) shows two major conformers. **(A)** Exchange cross-peaks at 5.5 ppm and 6.2 ppm indicate two long-lived, exchanging conformers. **(B)** Through-space magnetization shows both conformers exist simultaneously based on opposite-sign cross-peaks (blue).

The observability of any cross-peak in the TOCSY or ROESY spectra (Fig. 2 and Figs. S4-S6) necessarily indicates a conformational state that is well occupied on NMR time scale. Although we cannot assign these peaks, we can simply count the largest peaks and put a lower-bound on the minimum fraction of the total population in these. From spectral deconvolution of 1D spectra for **H4** peptoid we obtained approximately 24 doublets, which would correspond to 12 different conformational states; most of them present in very small quantities (see ESI†). Crucially, the ROESY intensity of the two main conformers comprises 80% of the total intensity observed, and the deconvolved 1D NMR intensity fraction is roughly 65%. This difference is consistent with the small fractions represented by several of the conformers (as deduced from 1D deconvolution) not being observed in the ROESY spectra with lower signal to noise. The ratio between the two main conformers by 1D NMR is approximately 2:1 (see SI). Further, the existence of exchange peaks for the major conformers indicate two long-lived peptoid conformers that interconvert. However, not every exchange peak is observable due to a combination of factors: 1) The exchange rates are outside of the range of the experimental modality, 2) overlapping spectra, 3) signals too close to the diagonal, or 4) signal is below the noise threshold.

An important bridge toward predictive design depends on closing the gap between models and experiments. Using Rosetta,[34,45] an efficient biomolecular structural sampling tool with MC capabilities and peptoid parameters,[45-47] we generated ensembles of the **H4** peptoid that involve initializing the ψ angle to 180°. Comparisons with NMR ensembles of **H4** peptoid confirms the anticipated *i to i+3* secondary structure with a stacking metric based on pairwise distances of six-membered rings.[48] Distances between proximal and distal rings in naphthyl side-chains (Fig. S16, ESI†), and the average of both values in each conformer (Fig. 3) describes two general conformer types: 'stacked' (3–6 Å), and 'unstacked' (6–10 Å), with all other conformers described by several minor peaks at naphthyl-naphthyl distances >10.0 Å. These two conformers account for 86% of the ensemble (27% stacked, 59% unstacked) and demonstrate that the accessible basins of attraction for the energy landscape are dominated by two conformers. The major finding here is that when ψ is initialized to 180°, Rosetta, like NMR, identifies two major conformers rather than a continuum of states (our precision is not sufficient to say if the larger Rosetta population corresponds to the more populated conformer in NMR).

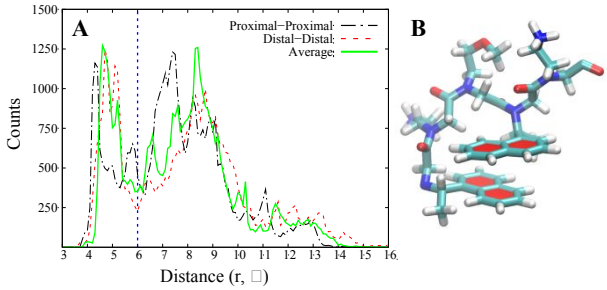


**Fig. 3.** **(A)** Naphthyl-naphthyl distances in peptoid **H4** divides the ensemble into preferred conformers consistent with NMR and CD results. Distances between naphthyl ring centers show well-defined peaks corresponding to 'stacked' (3 < r ≤ 6 Å) and 'unstacked/ extended' (6 < r ≤ 10 Å) conformers (separated by a dashed blue line). **(B)** The median centroid structure for the lowest energy conformers in the **H4** ensemble.

While it has long been assumed that π–π stacking of *i* to *i*+3 species was the critical component for reducing peptoid conformational flexibility, our results instead suggest that the reduced flexibility of the ψ backbone angle may be the determining factor for limiting peptoid conformational freedom (Fig. S17 & S19, ESI†).

In conclusion, this work represents a crucial step toward design of peptoid secondary structural elements, and their larger foldamers, with the chemical diversity required to mimic the complex functions of proteins (e.g. catalysis and molecular recognition). In addition to developing a rapid, economical synthetic procedure to generate

high-purity peptoid hetero-oligomers (required for downstream combinatorial production of peptoid foldamers from suites of unique secondary structural elements), we confirm through CD and NMR experiments, and molecular modelling, the significance of sequence on structural propensity. Based on CD and NMR spectroscopy interpretations, we observe a significant dependence of structural propensity on sequence. Detailed NMR analysis of the naphthyl-containing **H4** hetero-oligomeric peptoid indicates the ensemble is dominated by two major conformers, and Monte Carlo modelling recapitulates these results with the critical inclusion of a peptoid-specific bias ($\psi$ backbone angle initialization at 180°) (Fig. S17 & S18, ESI†). Model conformers of the **H4** peptoid ensemble can be represented by (1) a close naphthyl-naphthyl stacking arrangement, and (2) more extended conformations. Thus, incorporating peptoid-specific backbone parameters with naphthyl-naphthyl stacking is a successful and re-usable design principle for inducing constrained secondary structure in *individual, self-folding, water-soluble* hetero-oligomeric peptoids: a critical step towards synthesis of three-dimensional foldamers that mimic the natural chemical diversity found in nature.

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## Conflicts of interest

There are no conflicts of interest to declare.

1 R. J. Simon, R. S. Kania, R. N. Zuckermann, V. D. Huebner, A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C. K. Marlowe, *PNAS*, 1992, **89**, 9367.
2 N. Gangloff, J. Ulbricht, T. Lorson, H. Schlaad, R. Luxenhofer, *Chem. Rev.* 2016, **116**, 1753.
3 K. Kirshenbaum, A. E. Barron, R. A. Goldsmith, P. Armand, E.K. Bradley, K. T. V. Truong, K. A. Dill, F. E. Cohen, R. N. Zuckermann, *PNAS.* 1998, **95**, 4303.
4 M.L Huang, D. Ehre, Q. Jiang, C. Hu, K. Kirshenbaum, M. D. Ward, *PNAS.* 2012, **109**, 19922.
5 D. J. Murray, J. H. Kim, E. M. Grzincic, S. C. Kim, A. R. Abate, R. N. Zuckermann, *Langmuir.* 2019, **35**, 13671.
6 R. Gopalakrishnan, A. I. Frolov, L. Knerr, W. J. Drury, E. Valeur, *J. Med. Chem.* 2016, **59**, 9599.
7 L. M. S. Wolf, S. L. Servos, M. A. Moss, *J. Neurol. Neuromed.* 2017, **2**, 1.
8 J. Morimoto, et. al. *J. Am. Chem. Soc.* 2020, **142**, 2277
9 J. Sun, R. N. Zuckermann, *ACS Nano.* 2013, **7**, 4715.
10 R. N. Zuckermann, *J. Pept. Sci.* 2011, **96**, 545.
11 K. Huang, C.W. Wu, T. J. Sanborn, J. A. Patch, K. Kirshenbaum, R. N. Zuckermann, A. E. Barron, I. Radhakrishnan, *J. Am. Chem. Soc.* 2006, **128**, 1733.
12 T. S. Burkoth, E. Beausoleil, S. Kaur, D. Tang, F. E. Cohen, R. N. Zuckermann, *Chem. Biol.* 2002, **9**, 647.
13 A. W. Wijaya, A. I. Nguyen, L. T. Roe, G. L. Butterfoss, R. K. Spencer, N. K. Li, R. N. Zuckermann, *J. Am. Chem. Soc.* 2019, **141**, 19436.
14 C. W. Wu, T. J. Sanborn, K. Huang, R. N. Zuckermann, A. E. Barron, *J. Am. Chem. Soc.* 2001, **123**, 6778.
15 J. A. Patch, A. E. Barron. *J. Am. Chem. Soc.* 2003, **125**, 12092.
16 P. Armand, K. Kirshenbaum, R. A. Goldsmith, S. Farr-Jones, A. E. Barron, K. T. V. Truong, K. A. Dill, D. F. Mierke, F. E. Cohen, R. N. Zuckermann, E. K. Bradley, *PNAS.* 1998, **95**, 4309.
17 C. M. Darapaneni, P. J. Kaniraj, G. Maayan. *Org. Biomol. Chem.* 2018, **16**, 1480.
18 T. J. Sanborn, C. W. Wu, R. N. Zuckermann, A. E. Barron, *Biopolymers.* 2002, **63**, 12.
19 B. C. Gorske, B. L. Bastian, G. D. Geske, H. E. Blackwell, *J. Am. Chem. Soc.* 2007, **129**, 8928.
20 S. Hoyas, E. Halin, V. Lemaur, J. De Winter, P. Gerbaux, J. Cornil, *Biomacromolecules.* 2020, **21**, 903.
21 D. Gimenez, J.A. Aguilar, E. H. C. Bromley, S. L. Cobb, *Angew. Chem. Int. Ed.* 2018, **57**, 10549.
22 D. Gimenez, G. Zhou, M. F. D. Hurley, J. A. Aguilar, V. A. Voelz, S. L. Cobb, *J. Am. Chem. Soc.* 2019, **141**, 3430.
23 R. Armand, K. Kirshenbaum, A. Falicov, R.L. Dunbrack, K. A. Dill, R. N. Zuckermann, F. E.; Cohen, *Fold. Des.* 1997, **2**, 369.
24 C. W. Wu, K. Kirshenbaum, T. J. Sanborn, J. A. Patch, K. Huang, K. A. Dill, R. N. Zuckermann, A. E. Barron, *J. Am. Chem. Soc.* 2003, **125**, 13525.
25 G. Angelici, N. Bhattacharjee, O. Roy, S. Faure, C. Didierjean, L. Jouffret, F. Jolibois, L. Perrin, LC; Tallefumier, *Chem. Commun.* 2016, **52**, 4573.
26 O. Roy, G. Dumonteil, S. Faure, L. Jouffret, A. Kriznik, C. Taillefumier, *J. Am. Chem. Soc.* 2017, **139**, 13533.
27 M. Rzeigui, M. Traikia, L. Jouffret, A. Kriznik, J. Khiari, O. Roy, C. Taillefumier, *J. Org. Chem.* 2020, **85**, 2190.
28 V. A. Voelz, K. A. Dill, I. Chorny. *Biopolymers*. 2011, **96**, 639.
29 G. L. Butterfoss, P. D. Renfrew, B. Kuhlman, K. Kirshenbaum, R. Bonneau. *J. Am. Chem. Soc*. 2009, **131**, 16798.
30 J. Laursen,; P. Harris; P. Fristrup, C. A. Olsen. *Nat. Commun.* 2015, **6,** 7013.
31 J. A. Schneider, T.W. Craven, A.C. Kasper, et. al. *Nat. Comm.* 2018, **9**, 4396.
32 Roe, L.T., et. al. *Biopolymers*. 2019, **110**, e23267
33 G. J. Rocklin, T. M. Chidyausiku, I. Goreshnik, et. al. *Science.* 2017, **357**, 168.
34 C. A. Rohl, C. E. M. Strauss, K. M. S. Misura, D. Baker, *Methods in Enzymology*, Academic Press, 2004.
35 R. K. Jha, T. Gaiotto, A. R. M. Bradbury, C. E. M. Strauss, *Protein Eng. Des. Sel.* 2014, **27**, 127.
36 R. K. Jha, S. Chakraborti, T. L. Kern, D. L. Fox, C. E. M. Strauss, *Proteins.* 2015, **83**, 1327.
37 R. N. Zuckermann, J. M. Kerr, S. B. H. Kent, W. H. Moos, *J. Am. Chem. Soc.* 1992, **114**, 10646.
38 G. M. Figliozzi, R. Goldsmith, S. C. Ng, S. C. Banville, R. N. Zuckermann, *Methods in Enzymology*, Academic Press, 1996.
39 C. O. Kappe, *Angew. Chem. Int. Ed.* 2004, **4**, 6250.
40 H. J. Olivos, P. G. Alluri, M. M. Reddy, D. Salony, T. Kodadek, *Org. Lett.* 2002, **4**, 4057.
41 B. C. Gorske, S. A. Jewell, E. J.; Guerard, H. E. Blackwell, *Org. Lett.* 2005, **7**, 1521.
42 Shin, H.-M.; Kang, C.-M.; Yoon, M.-H.; Seo, J. *Chem. Commun.* 2014, **50**, 4465.
43 Stringer, J. R.; Crapster, J. A.; Guzei, I. A.; Blackwell, H. E. *J. Am. Chem. Soc.* 2011, **133**, 15559.

44 Fuller, A. A.; Yurash, B. A.; Schaumann, E. N.; Seidl, F. *J. Org. Lett.* 2013, **15**, 5118.
45 Leaver-Fay, A.; et. al. *Methods in Enzymology*, Johnson, M. L.; Brand, L., Eds. Academic Press: 2011; Vol. 487, 545.
46 P. D. Renfrew, T. W. Craven, G. L. Butterfoss, K. Kirshenbaum, R Bonneau, *J. Am. Chem. Soc.* 2014, **136**, 8772.
47 K. Drew, et. al. *PLOS ONE.* 2013, **8**, e67051.
48 J. C. Miner and A. E. García, *J. Phys. Chem. B.* 2017, **121**, 3734.