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Synthesis and Self-Assembly of Bundle-Forming α -Helical Peptide–Dendron Hybrids

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Abstract

Dendronized helix bundle assemblies combine the sequence diversity and folding properties of proteins with the tailored physical properties of dendrimers. Assembly of peptide-dendron hybrids into α -helical bundles encapsulates the helix bundle motif in a dendritic sheath that will allow the functional, protein-like domain to be transplanted to non-biological environments. A bioorthogonal graft-to synthetic strategy for preparing helix bundle-forming peptide-dendron hybrids is described herein for hybrids **1a**, **1b**, and **2**. Titration experiments monitored by circular dichroism spectroscopy support our self-assembly model for how the peptide-dendron hybrids self-assemble into α -helical bundles with the dendrons on outside of the bundle.

Keywords

Peptides; dendrimers; self-assembly; coiled-coil

Introduction

Control over polymer chain length, monomer sequence, and chain folding are essential to the functionality of natural biopolymers (e.g., proteins, DNA, RNA). Dendrimers¹ and β -peptide foldamers² are among the best examples of synthetic macromolecules to rival natural biopolymers. These materials, however, highlight the challenges to bridging the gap between controlled structure and function. De novo protein design and protein engineering strategies, on the other hand, exploit natural building blocks to create protein mimetics that perform natural and non-natural functions.³ Considering the limited range of properties available to natural protein materials, hybrid materials are a promising route to combine desirable properties of both natural and synthetic materials.⁴

Peptide-dendron conjugates^{6–12} are hybrid materials in which synthetic chemistry provides complete control over macromolecular structure and chain folding can be regulated through self-assembly. A variety of morphologies have been observed from peptide-dendron hybrids

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Associated Content: Supporting Information. The Supporting Information is available free of charge on the ACS Publications Website.

Chemical drawings depicting the structure of each peptide-dendron hybrid and peptide-tetraethylene glycol conjugate, tables enumerating the peptide synthesis operations, HPLC chromatograms and MALDI-TOF spectra of new compounds.

that self-assemble due to backbone-backbone hydrogen bonding interactions.^{6a,6b,12} It is possible to interconvert between some of these morphologies in response to external stimuli,^{6b,12a,12b} and to program their polymerization and depolymerization.^{7d} Functional supramolecular materials such as organogels,^{7a,7b} porous channel protein mimetics,⁸⁻⁹ and switchable channels^{7c} illustrate the advantages of hybrid materials to address long-standing challenges in supramolecular materials. These abiotic materials can perform biological functions in biologically relevant environments,^{8,10} and have inspired curiosity about the origins of homochirality in nature.^{9h,13,14}

We recently described a helix bundle-forming peptide-dendron hybrid (**1a**, Scheme 1).¹⁵ Helix bundle motifs in proteins support diverse functions such as catalysis in water-soluble enzymes^{4b} and ion/small-molecule transport among transmembrane proteins¹⁶ due, in part, to the well defined arrangement of amino acid side chains in the core of the bundle. Our long-term goal is to encapsulate helix bundles in a dendritic sheath that will allow us to deploy functional helix bundle nanostructures in non-biological environments. Here we provide a detailed account of our efforts to design, synthesize, and characterize helix bundle-forming peptide-dendron hybrids **1a**, **1b**, and **2** (Scheme 1).

Experimental Section

Materials

Trifluoroacetic acid (TFA) was used as received from Aldrich. Anhydrous *N,N*-dimethylformamide (DMF), sodium azide, and Fmoc-Tyr(*t*-Bu)-OH were used as received from EMD. Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Pra-OH, *N*-methylmorpholine (NMM), and *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluroniumhexafluorophosphate (HBTU) were used as received from Chem-Impex International. Acetonitrile (MeCN) was used as received from Fisher. Triisopropylsilane and copper(II) sulfate pentahydrate were used as received from Acros. L-Ascorbic acid sodium salt and acetic anhydride (Ac₂O) were used as received from Alfa-Aesar. Methanol (MeOH) and dimethylsulfoxide (DMSO) were used as received from BDH. H-Rink amide-ChemMatrix resin (0.51 mmol/g) was used as received from PCAS BioMatrix Inc. Isopropyl alcohol was used as received from Pharmaco-Aaper. Potassium chloride was used as received from G-Biosciences.

Tris(hydroxymethyl)aminomethane hydrochloride (Tris•HCl) and

tris(hydroxymethyl)aminomethane (TRIS) were used as received from Amresco.

Compounds **6-11**, peptide-dendron hybrid **1a**, peptide **3a** and Ac-YLKLLKLLKLLK-NH₂ (**5a**) were prepared according to literature procedures.¹⁷ Peptides were assembled by manual^{17b} or automated¹⁵ solid-phase peptide synthesis according to general protocols.

Peptide concentration and salt titration CD experiments were done according to previously described general protocols.¹⁵

Techniques

Peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on a Protein Technologies PS3 peptide synthesizer. High-performance liquid chromatography (HPLC) was performed on a Waters 1525 Binary pump equipped with a Waters 2489 UV/Visible Detector set at 210 nm and 280 nm. To determine the analytical purity of samples, the

HPLC was equipped with a SunFire™ C18 column (5 μ m, 4.6 mm $\times$ 250 mm; Waters) operating at a flow rate of 1 mL/min and a solvent gradient of 1%/min. Purification of samples was done on the HPLC equipped with a SunFire™ C18 (5 μ m, 19 mm $\times$ 250 mm) column operating at a flow rate of 17 mL/min and a solvent gradient of 5%/min. Samples for MALDI-TOF MS were prepared using α -cyano-4-hydroxycinnamic acid as the matrix. Equal volumes of solutions (10 mg/mL) of matrix and peptide in MeCN/H₂O (1:1 v/v) were mixed. The mixture of matrix and analyte (1 μ L) was deposited on the target plate and allowed to dry before measurements were taken. Absorbance of peptide solutions in deionized water at 280 nm was recorded on an Evolution 201 spectrophotometer using a 1-cm quartz cuvette. The concentration of the peptide was calculated from the absorbance using Beer's Law and an extinction coefficient (ϵ) for tyrosine of 1490 L mol⁻¹ cm⁻¹.¹⁸ Circular dichroism (CD) spectra were recorded on an Applied Photophysics Chirascan instrument.

Assembly of Ac-YLPraKLLKLLPraKLLKLLPraKLLK-NH₂ (3b)

Peptide **3b** was assembled on ChemMatrix Rink amide resin (0.2096 g, 0.1069 mmol) in a glass-fritted peptide reaction vessel using a PS3 synthesizer and cleaved from the resin to yield peptide **3b**. The sequence of operations is detailed in Supporting Information (Table S1).

Assembly of Ac-YLPraKLLKLLKPraLLK-NH₂ (4)

Peptide **4** was assembled using a PS3 automated synthesizer on ChemMatrix Rink amide resin (0.1937 g, 0.09879 mmol) in a glass-fritted peptide reaction vessel and cleaved from the resin to yield peptide **4**. The sequence of operations is detailed in Supporting Information (Table S2).

Assembly of Ac-YLPraKLLKLLKPraLLK-NH₂ (4)

Peptide **4** was assembled manually on ChemMatrix Rink amide resin (0.2511 g, 0.1280 mmol) in a glass-fritted peptide reaction vessel and cleaved from the resin to yield peptide **4**. The sequence of operations is detailed in Supporting Information (Table S3).

Assembly of Ac-YLKLLKLLKLLKLLKLLK-NH₂ (5b)

Peptide **5b** was assembled using a PS3 automated synthesizer on ChemMatrix Rink amide resin (0.2567 g, 0.1309 mmol) in a glass-fritted peptide reaction vessel and cleaved from the resin to yield peptide **5b**. The sequence of operations is detailed in Supporting Information (Table S4).

Peptide-Dendron Hybrid 1b

Dendritic azide **11** (16.4 mg, 15.0 μ mol) was dissolved in 200 μ L H₂O and transferred to a vial containing peptide **3b** (7.6 mg, 3.0 μ mol) in 300 μ L of DMSO. The reaction mixture was purged with N₂ for one hour prior to adding CuSO₄·5H₂O (0.6 mg, 2 μ mol) and sodium ascorbate (1.2 mg, 6.1 μ mol) in 100 μ L H₂O. The reaction mixture was stirred at room temperature under a N₂ atmosphere for 17 h. The reaction mixture was purified by preparative HPLC to yield **1b** (8.3 mg, 47%) as a colorless powder.

Peptide-Dendron Hybrid 2

Dendritic azide **11** (10.2 mg, 9.30 μmol) was dissolved in H_2O (110 μL) and transferred to a vial containing peptide **4** (5.5 mg, 3.2 μmol), a premixed solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.2 μL , 1 M solution in H_2O), and sodium ascorbate (2 mg, 0.01 mmol) in DMSO (110 μL). The reaction mixture was stirred at room temperature under a N_2 atmosphere for 90 h. The reaction mixture was purified by preparative HPLC to yield **2** (6.7 mg, 53 %) as a colorless powder.

Peptide-Linear Hybrid 16

Azide **15** (7.8 mg, 0.034 mmol) was dissolved in 150 μL 1 M TRIS-HCl buffer (pH 8) and transferred to a vial containing peptide **3a** (6.8 mg, 0.0040 mmol), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.2 mg, 4.8 μmol), and sodium ascorbate (2.5 mg, 0.013 mmol) in 150 μL of DMSO. The reaction mixture was stirred at room temperature under a N_2 atmosphere for 15 h. The reaction mixture was purified by preparative HPLC to yield **16** (3.8 mg, 44%) as a colorless powder.

Peptide-Linear Hybrid 17

Azide **15** (5.1 mg, 2.3 μmol) was dissolved in 1 M TRIS-HCl buffer (pH 8) (150 μL) and transferred to a vial containing peptide **4** (5.0 mg, 2.9 μmol), a premixed solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.5 μL , 1 M solution in H_2O), and sodium ascorbate (1.2 mg, 6.1 μmol) in DMSO (150 μL). The reaction mixture was stirred at room temperature under a N_2 atmosphere for 15 h. The reaction mixture was purified by preparative HPLC to yield **17** (1.5 mg, 22%) as a colorless powder.

Results and Discussion

Design Rationale

Bundle-forming α -helical peptide-dendron hybrids were designed by site-specifically introducing water-soluble dendrons at solvent-exposed, surface residues of the helix bundle assembly. A design principle that is common to all water-soluble helix bundle-forming peptides is that polar and non-polar amino acid residues are clustered on opposite sides of the α -helix so that an amphipathic structure is produced upon folding of the peptide.¹⁹ Sequestration of the hydrophobic residues at the core of the bundle provides the driving force for assembly of the α -helical peptides. Further design considerations such as the choice of residues that form the core of the bundle, the types of interactions at the interface between helices, and placement of molecular recognition motifs within the core of the bundle improve the specificity of the design.¹⁹ Nonetheless, patterning of polar and non-polar residues in the amino acid sequence to match the periodicity of the α -helical fold in a bundle (Scheme 2) can be sufficient to design a bundle-forming peptide.²⁰ Residues on the exterior of the bundle minimally contribute to the folding and assembly of helix bundle-forming peptides, which makes these residues convenient sites to which prosthetic groups can be conjugated. Indeed, polymers,²¹ catalysts,²² and other groups²³ have been appended to exterior positions of helical bundles.

Leucine and lysine were chosen as the hydrophobic (h) and polar (p) amino acids, respectively. Several leucine-lysine (a.k.a., "LK") peptides that form α -helical bundles have

been designed by following the pattern (hpphhph)₂.^{20,24} Notably, the groups of DeGrado and Lear^{20a} and Shaw^{20b} have independently shown that “LK” peptides as short as fourteen residues form α -helical bundles made up of only four or five helices. Our designed peptide^{17b} follows a similar pattern (hhpphhp)₂ with the polar residues more central to the heptad (Scheme 2a). The *N*-terminus was acetylated and the *C*-terminus was amidated to maximize α -helix propensity of the peptide by replacing ionizable groups with hydrogen bonding groups.²⁵ The *N*-terminal hydrophobic residue was assigned as tyrosine rather than leucine in order to have a spectroscopic probe with which we could determine the concentration of the peptide-dendron hybrid in solution.¹⁸ Two of the polar residues were selected as sites for conjugating hydrophilic dendrons to the peptide.

A single hydrophilic dendron was positioned in each heptad of the bundle-forming peptide-dendron hybrid. A seven-residue repeat encodes the helix bundle motif, and three of these residues are polar, surface residues in our designed α -helical bundle. We chose residue-3 in the first heptad of the amino acid sequence as the position for the first dendron. Positioning the dendrons at surface residues following an *i, i + 7* periodicity matches the natural periodicity of the α -helix. Residues at positions *i* and *i + 7* are in register with each other when viewed along the long axis of the α -helix (Scheme 2d), so we placed the second dendron at residue-10 in peptide-dendron hybrid **1a**. This arrangement was anticipated to minimize contact between the dendrons on one helix with the other α -helical peptide-dendron hybrids in the bundle. On the other hand, having the dendrons in register along the α -helix increases the likelihood for intramolecular interactions between the dendrons that could destabilize the α -helical folded state of the hybrid. We, therefore, designed a second peptide-dendron hybrid **2** wherein the dendrons are spaced *i, i + 8* (Scheme 2d).

Water-soluble poly(alkyl ether) dendrons were chosen to conjugate to the helix bundle because these dendrons would facilitate circular dichroism (CD) spectroscopy experiments to characterize the folding and self-assembly of the peptide-dendron hybrids. Chromophores in a chiral environment differentially absorb left- and right-handed circularly polarized light. The chromophores of interest in peptides are the backbone amide groups, which can be obscured by other amide or aromatic chromophores in the molecule.²⁶ While several groups have circumvented this complication through combinations of spectroscopies,⁶⁻⁹ we preferred to identify dendrons that would not interfere with our interpretation of the CD spectra. Furthermore, it was important that the water-soluble dendrons be achiral so that the dendrons do not contribute to the asymmetric environment of the amide chromophores. The dendrons in hybrids **1a** and **2** are composed of symmetrically branched alkyl ether units that do not absorb UV light in the same region as the amides.

The dendrons are linked to the peptide through 1,4-triazole groups that are introduced in copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. While the diversity of amino acids in peptide-dendron hybrids **1** and **2** is intentionally limited, we sought a synthesis strategy that would be compatible with a wide range of amino acids. The CuAAC reaction is bioorthogonal,²⁷ and introduces a relatively small side-chain functionality. Because the CuAAC reaction can be performed in mixed aqueous/organic solvent and is compatible with the functional groups of most natural amino acids, the 1,4-triazole linkages make it feasible to attach the dendrons to the peptides in a graft-to manner. That is to say

that the dendrons and peptide can be synthesized and purified to homogeneity using standard techniques before attempting the synthesis of the hybrids. This represents a highly convergent synthesis strategy and ensures that the products of the CuAAC reaction are free of any structurally defective impurities.

Synthesis of the Alkyne-Containing Peptides

Alkyne-containing peptides **3a** and **4** were prepared following manual^{17b} or automated¹⁵ solid-phase peptide synthesis protocols. Commercially available Fmoc-Pra-OH (Pra = propargylglycine) was coupled to the peptide at positions in the amino acid sequence where the hydrophilic dendrons were to be conjugated. The assembled peptides were cleaved from the resin and purified to homogeneity by HPLC. The suitability of **3** and **4** to preparation and purification by standard peptide chemistry protocols illustrates the versatility of peptides as sequence defined macromolecular building blocks.

An optimized protocol for the manual synthesis of peptide **5a** was developed. Peptide **5a** is a control peptide in which all of the surface residues are lysines. Manual solid-phase peptide synthesis allows us to monitor the deprotection of each amino acid coupled to the resin, which indirectly reports on the efficiency of each coupling reaction. We judged the overall success of the synthesis from analytical HPLC traces of the crude products. Figure 1 shows representative HPLC data for syntheses on three different solid-phase resins: a high-loading polystyrene (PS) resin (0.71 mmol/g), a low-loading PS resin (0.38 mmol/g), and a polyethylene glycol (PEG) resin (0.51 mmol/g). Low purity of crude peptides can be attributed, in part, to collapse of the growing peptide chain due to hydrophobic effects and/or hydrogen bonding between growing chains during peptide assembly. Hydrophobic resins like PS can exacerbate aggregation of growing peptides. Reducing the loading of the resin and the effective concentration of growing chains has been reported to increase the crude purity of peptides.²⁸ The polar PEG resin has gained attention for yielding very clean crude peptides compared to high-loading PS resins.^{28c,29} Because the PEG resin swells much more than PS resin in the reaction solvent (i.e., DMF), we included the low-loading PS resin to assess the extent to which the effective concentration of growing peptide chains affects the purity of the crude peptide products. The data in Figure 1 show that the crude product from the high-loading PS resin is significantly less pure than the crude products from the low-loading PS and PEG resins. The effective concentration of growing chains on the resin appears to be the major determinant of the purity of crude peptide **5a**, and similar results were later obtained for peptide **4**. Because more peptide is obtained per mass of resin from the PEG resin than the low-loading PS resin, we prefer the PEG resin rather than the low-loading PS resin.

Manual synthesis of peptide **4** presented challenges that were not encountered in the synthesis of **5a**. When we synthesized **4** on high-loading PS resin, we found that several amino acids required multiple iterations of the deprotection protocol to completely remove the *N*-terminal protecting group and observed a steep decline of the coupling efficiencies for residues 1-7 of the amino acid sequence. These symptoms are associated with so-called “difficult sequences,”³⁰ and can be attributed to the added hydrophobicity of the amino acid sequence when lysine residues are replaced with propargylglycine residues. One defect

structure was especially troubling. Poor coupling of Leu5 resulted in a truncated peptide that co-elutes with **4** from the HPLC column. The structure of the co-eluting peptide was identified from a tandem mass spectrometry experiment.³¹ Changing the resin to the PEG resin alleviated most of the difficulties in the synthesis of **4**, however we continued to double couple Leu5 to avoid any contamination from the co-eluting impurity.

Adaptation of the manual solid-phase synthesis protocol to an automated system was straightforward, and worked equally well for peptides **3a** and **4**. A single coupling reaction was required to introduce each amino acid to the resin except for Leu5; a double coupling was used to introduce Leu5. The protocol was also extended to the synthesis of the 21-residue peptides **3b** and **5b**. Peptide **3b** required double couplings at Leu5 and Leu12, and peptide **5b** required double couplings at Leu6 and Leu13. The key differences between the manual^{17b} and automated syntheses¹⁵ have to do with the coupling reaction conditions. In the manual synthesis the *N*^α-Fmoc-protected amino acids are activated with HBTU/HOBt and diisopropylethylamine as the base ([HBTU]₀/[HOBt]₀/[DIEA]₀/[Fmoc-AA-OH]₀ = 0.9/1/2/1; [Fmoc-AA-OH]₀ = 0.5 M). The automated synthesis employs HBTU with *N*-methylmorpholine as the base ([HBTU]₀/[HOBt]₀/[NMM]₀/[Fmoc-AA-OH]₀ = 0.9/0/0.8/1; [Fmoc-AA-OH]₀ = 0.2 M). The coupling reaction times were shorter for the automated synthesis than the manual synthesis (20 min vs. 40 min). Capping with Ac₂O was performed after each amino acid was coupled to the resin to prevent chain extension of any defective peptides. The conditions for removing the *N*-terminal protecting group and for cleaving the peptide from the resin with simultaneous side-chain deprotection were identical for the manual and automated syntheses. After purification by reversed-phase HPLC, the identity and purity of the peptides were confirmed by MALDI-TOF mass spectrometry and analytical HPLC, respectively.

Synthesis of the Dendritic Azide

A water-soluble, second-generation dendron **11** was prepared by surface functionalization^{17a,32} of poly(alkyl ether) dendron **10** (Scheme 4). As demonstrated by Fréchet and co-workers,^{17a,32} the surface functionalization strategy combines the precision of convergent dendron syntheses with the structural diversity of end groups that is usually reserved for dendrimers prepared in the divergent manner. A single intermediate dendron can be transformed into materials with diverse physical properties.^{17a,32}

The second-generation poly(alkyl ether) dendron **8** was prepared in the convergent manner developed by Fréchet and co-workers.^{17a,32-33} Acid-catalyzed condensation of tris(hydroxymethyl)ethane with 5-nonanone in refluxing benzene provided the ketal-protected, first-generation dendron **6**. Dietherification of methallyl dichloride with **6** under Williamson conditions afforded the second-generation alkene **7**. We have previously noted that 15-crown-5 improves the efficiency of the generational growth step in the synthesis of poly(alkyl ether) dendrons,³⁴ and this modification was employed in the synthesis of **7**. Dendritic alcohol **8** was obtained from hydroboration-oxidation of **7**. As mentioned by Grayson and Fréchet,^{17a} the solubility and polarity of the ketal-protected dendrons **6-8** facilitate the synthesis and purification of these dendrons on a multigram (>10-g) scale.

Dendron **10** with orthogonally reactive apex and peripheral groups was prepared in three steps from alcohol **8**. Treatment of **8** with mesyl chloride and pyridine in CH₂Cl₂ affords a mesylate intermediate that was isolated and used without further purification. Nucleophilic substitution of the mesylate with sodium azide introduced the desired azide group at the apex of the dendron for CuAAC reactions. A solid-supported acid catalyst was employed to effect hydrolysis of the ketal groups^{17a,32} in **9**, and which made it possible to isolate **10** by filtration to remove the resin and evaporation of the solvent.

Exhaustive surface functionalization of the peripheral hydroxyl groups of poly(alkyl ether) dendrons such as **10** can be achieved with a range of electrophiles. Fréchet and co-workers have shown that acyl chlorides, *O*-acylisoureas, allylic and benzylic halides, and alkyl tosylates are all sufficiently good electrophiles to effect quantitative reaction with each of 16 surface hydroxyl groups on a fourth-generation dendron with alkene apex functionality.^{17a,32} Etherification of **10** with the tosylate of tetraethylene glycol monomethyl ether was quantitative, which highlights the orthogonality of the azide and hydroxyl groups. Dendritic azide **11** was obtained in 86% yield after chromatographic purification.

The surface functionalization approach proved especially valuable in the synthesis of a water-soluble dendritic azide for conjugation to peptides. Dendron **10** efficiently undergoes CuAAC reactions with dipeptides and peptide **4** to yield peptide-dendron hybrids that were soluble in aqueous/organic solvent mixtures but not in aqueous buffer. Surface functionalization of dendron **10** with tetraethylene glycol units was required to obtain a peptide-dendron hybrid that is soluble in aqueous buffer. We also investigated syntheses of **13** and **14** (Scheme 5). Chemoselective debenzoylation of **12**^{34a} under oxidative conditions³⁵ yielded **13** but the product was difficult to isolate from the reaction mixture. An attempt to synthesize **14** in a fully convergent manner similar to that described by Cho and co-workers³⁶ was abandoned because tedious chromatographic purifications were needed to obtain dendrons of acceptable purity. Dendron **11**, on the other hand, was readily prepared in good overall yield.

Grafting Dendrons to the Peptides

A graft-to strategy for synthesizing the α -helical bundle-forming peptide-dendron hybrids should minimize the number of impurities that need to be removed during purification. Conjugating the dendrons to a deprotected peptide avoids having to perform additional chemical reactions and purification operations on the hybrid materials. The hydrophilicity of the dendrons made it convenient to purify the peptide-dendron hybrids by reversed-phase HPLC. Defect-free hybrids, such as those obtained through this strategy, enable a detailed understanding of the structure-property relationships in architecturally complex macromolecules.

Ligand-free copper-catalyzed azide-alkyne cycloaddition (CuAAC) of dendron **11** with peptides **3** and **4** yielded peptide-dendron hybrids **1** and **2**, respectively (Scheme 6). Ligands are usually employed for bioconjugation by CuAAC to compensate for slow reaction kinetics at low substrate concentrations,^{27,37} to stabilize the catalytically active Cu(I)-species against oxidation,^{37b,38} and to suppress side reactions with certain amino acids (e.g.,

histidine).^{38a,38c} High concentrations (>1 mM) of **3**, **4**, and **11** were achieved in DMSO/H₂O mixtures (1:1 v/v). Substoichiometric amounts of Cu(I) (0.2 eq. per alkyne) with the high concentrations of the substrates afforded fast reactions. Although the reaction mixtures were not degassed, the reactions were performed under an N₂ atmosphere and sodium ascorbate (4 eq. per Cu) was used as an *in situ* reducing agent to prevent accumulation of Cu(I) before the reactions were complete. CuAAC Reactions with **3b** required deoxygenated solvents to achieve complete conversion. The reactions were monitored by MALDI-TOF mass spectrometry. We observed complete disappearance of the starting peptide and monodendritic peptide-dendron hybrid intermediate without any evidence for copper-mediated macrocyclization or oligomerization of the peptides.

MALDI-TOF Mass spectrometry and analytical reversed-phase HPLC experiments confirmed the identity and purity of the peptide-dendron hybrids. The MALDI-TOF mass spectra shown in Figure 2 support that we have successfully isolated peptide-dendron hybrids **2** and **1b** from any residual dendron, starting peptide, or partially dendronized intermediates. We observe three different ionized forms of **2** ([M + X]⁺, X = H, Na, and K). We see excellent agreement between the observed isotope pattern for the peak assigned as the [M + H]⁺ ion to the expected isotope pattern for the same ion (Figure 2 insets). The attributes of the MALDI-TOF mass spectrum emphasize that we have synthesized the desired peptide-dendron hybrid as a monodisperse macromolecule with a completely defined chemical structure. Similar results for peptide-dendron hybrid **1a** were reported previously.¹⁵

Azides **11** and **15** revealed a stark dependence on solvent in their CuAAC reactions. When the reaction of peptide **4** with linear azide **15** was attempted in DMSO/H₂O (1:1 v/v), we obtained a mixture of starting peptide, monotriazole intermediates, and ditriazole product. We speculated that trifluoroacetic acid bound to peptide **4** might interfere with the CuAAC reaction as some ligand-assisted CuAAC reactions are sensitive to pH.⁴⁰ Typical conditions for preparing bioconjugates via the CuAAC reaction employ buffered aqueous solvent (or co-solvent) rather than H₂O.^{27,37-38} Because concentrated solutions of peptide **5** (i.e., 0.5 M) are stable in TRIS buffer, we attempted CuAAC reactions in DMSO/TRIS (pH 8) (1:1 v/v). Linear azide **15** reacts cleanly with both peptides **3** and **4** in buffered solvent mixture whereas the dendritic azide **11** yields a mixture of starting peptide, monotriazole intermediates, and ditriazole product. We further note that the same batch of peptide **4** that reacted incompletely with **15** underwent a clean reaction with **11** in DMSO/H₂O (1:1 v/v). These results demonstrate that residual trifluoroacetic acid is not the source of observed slow reaction kinetics. While the different reactivity of azides **11** and **15** is hard to reconcile, these experiments highlight the benefit of CuAAC reactions being “wide in scope”⁴¹ with respect to the range of solvent conditions under which the reaction exhibits high fidelity.

Folding and Self-Assembly

Circular dichroism (CD) spectra used to characterize the secondary structure of the peptides were found to depend on the peptide concentration and ionic strength of the solutions. The unfolded–folded and monomer–*n*-mer equilibria are coupled (Figure 3a) so we measure the extent to which bundles have formed on the basis of the intensity of the α-helix signature in

the CD spectrum. CD Spectra of α -helical peptides are characterized by minima at 208 and 222 nm. We used the mean residue ellipticity at 222 nm ($[\theta]_{222}$) to monitor the formation of α -helical bundles of peptide-dendron hybrids **1a** and **2** as well as peptide **5a** and conjugates **16** and **17**. From a series of titration experiments in which the peptide concentration or chloride concentration were varied, we identified conditions under which peptide **5a**, peptide-dendron hybrids **1a** and **2**, and conjugates **16** and **17** were maximally folded and assembled as α -helical bundles (Figure 3).

Plots of $[\theta]_{222}$ versus the concentration of peptide demonstrate that each compound cooperatively self-assembles into a bundle of α -helices. The CD spectra for monomeric α -helical structures would be independent of the concentration of compound. The observed dependences confirm that our designed, α -helical “LK” peptide (**5a**) creates an amphipathic structure upon folding. Furthermore, the dramatic change in $[\theta]_{222}$ at concentrations of **5a** below 50 μ M is indicative of a cooperative folding process. Similar features are observed in the titrations of **1a**, **2**, **16**, and **17** (Figure 3b,c). Replacing polar surface residues with hydrophilic dendrons (**1a** and **2**) or linear tetraethylene glycol moieties (**16** and **17**) does not interfere with the folding and self-assembly program encoded in the primary structure (i.e., amino acid sequence) of the peptide.

The high density of cationic residues makes α -helical “LK” peptides sensitive to chloride ion concentration.^{20a} Increasing ionic strength shields the cationic charges. Because the cationic residues are clustered together on one face of the amphipathic α -helix formed by these peptides, increasing ionic strength stabilizes the helix bundle assembly. Peptide **5a** exhibits the strongest dependence on chloride ion concentration among the compounds in Figure 3d,e. Peptides **3a** and **4** have two fewer lysine residues on the surface compared to **5a**. Because we conjugated non-ionic, hydrophilic groups to these surface positions, the overall number of charged sites on **1a**, **2**, **16**, and **17** are fewer than the number of cationic charges on **5a**. We reason that the larger number of cationic surface groups makes **5a** more sensitive to chloride concentration than the other compounds.

Spacing of Dendrons Along the α -Helix

Comparison of the CD spectra of peptide-dendron hybrids **1a** and **2** with that of peptide **5a** reveals that the hybrids are significantly less α -helical. The CD spectra in Figure 4 were recorded under conditions where each of the compounds is maximally folded, so the intensity of each spectrum directly reports on the effect of the changes to the surface residues. We calculated from $[\theta]_{222}$ values in each spectrum that hybrid **1a** is 76% as α -helical as **5a**¹⁵ and hybrid **2** is 63% as helical as **5a**. A significant contribution to the decreased helicity comes from steric interactions between the dendrons, especially between the peripheral groups.

The CD spectra of **1a** and **16** clearly show that the size of the dendron destabilizes the α -helical bundle (Figure 4a). In conjugate **16** a linear tetraethylene glycol chain replaces the dendron in **1a**. Conjugate **16** is as well folded as peptide **5a** which shows that the 1,4-triazolyl linkage introduced by the CuAAC reaction does not dramatically decrease the helicity of the conjugate. The decreased intensity of the CD spectrum of **1a** compared to

peptide **5a** and conjugate **16** is directly attributable to the structure of the dendron. Considering the large distance between the dendrons on each helix, we attribute the decreased helicity to the water-solubilizing peripheral groups on the dendron.

Each of the hybrids (**1a** and **2**) and conjugates (**16** and **17**) self-assemble into α -helical bundles, and those observations suggest that the self-assembled α -helical bundle is a resilient framework for building larger hybrid macromolecular assemblies. Comparing the CD spectra of **5a**, **16**, and **17** reveals that there are sequence-dependent factors that determine how well folded is each conjugate. Conjugates **16** and **17** have relatively small tetraethylene glycol groups conjugated at surface positions on the bundle that are spaced $i, i + 7$ and $i, i + 8$, respectively. Such a small change in sequence reduces the observed helicity by 17%. Compounding the observed sequence-dependent effect with the steric effect from large dendrons conjugated to the surface positions of the α -helical bundle would be expected to greatly reduce the helicity of **2** compared to **1a**. Indeed, hybrid **2** is less helical than **1a**.

Length of the Peptide

Increasing the length of the peptide has been shown to stabilize self-assembled α -helical bundles.⁴² The $i, i + 7$ spacing in **1** places the dendrons in register with each other along the length of the α -helix, and $i, i + 7$ periodicity can be propagated over longer peptides without interfering with the bundling of the α -helices. Increasing the length of the peptides from 14 residues (**1a**) to 21 residues (**1b**) did not improve α -helical structure on the basis of CD spectra (Figure 4c). It is important to note that the CD spectra from peptides **5a** and **5b** were essentially identical under these conditions.

Conclusion

α -Helical bundles are a privileged motif in natural proteins that provide a defined three-dimensional arrangement of side-chain functional groups necessary to achieve highly efficient and selective functionality. Transplanting α -helical bundle motifs into hybrid macromolecules offers a pathway for transplanting protein-like structure and function into non-biological environments. Dendronized α -helical bundles encapsulate the protein-like structure in a dendritic sheath that insulates the delicate structure of the folded, protein-like core. The peripheral groups of the dendrons can be varied in a modular fashion to tailor the compatibility of the dendronized helix bundle to different environments. We have described a convergent bioorthogonal strategy for conjugating dendrons site-specifically to peptides. Grafting dendrons at a density of one dendron per heptad along the hydrophilic face of an amphipathic α -helical peptide yields α -helical bundle-forming peptide-dendron hybrids. These new peptide-dendron hybrids are of fundamental interest to investigate the role of polymer architecture on folding of peptide-polymer conjugates, and may exhibit advantages over polydisperse polymer-peptide conjugates in applications such as drug delivery.⁴³ With a folded, protein-like core, the dendronized α -helical bundles described herein set a precedent for integrating other protein functions (e.g., catalysis) into supramolecular dendrimers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Chromatograms of the crude reaction product **5a** (solid line) obtained from syntheses on (a) high-loading PS resin, (b) low-loading PS resin, and (c) PEG resin. The dashed line indicates the solvent composition during elution of the peptide at 1 mL/min from a C18 column (solvent A = H₂O + 0.1% TFA; solvent B = MeCN/H₂O (9:1 v/v) + 0.1% TFA).

Figure 2.

MALDI-TOF Mass spectra of (a) **2** and (b) **1b**. The inset in each panel compares the simulated isotope pattern³⁹ (upper) with the observed isotope envelope (lower) for the $[M + \text{H}]^+$ ions (**2**: $\text{C}_{187}\text{H}_{352}\text{N}_{25}\text{O}_{60}$; **1b**: $\text{C}_{278}\text{H}_{525}\text{N}_{37}\text{O}_{89}$).

Figure 3.

(a) Schematic showing that folding and self-assembly are coupled equilibria. (b and c) Plots of $[\theta]_{222}$ versus the concentration of **1a** (○), **16** (△), **5a** (□), **2** (●), and **17** (▲). (d and e) Plots of $[\theta]_{222}$ versus chloride concentration for **1a** (○), **16** (△), **5a** (□), **2** (●), and **17** (▲).

Figure 4.

Circular dichroism (CD) spectra from 500 μ M solutions of (a) **1a** ($\circ$), **16** ($\triangle$), and **5a** ($\square$), (b) **2** ($\bullet$), **17** ($\blacktriangle$), and **5a** ($\square$), and (c) **1a** ($\circ$), **1b** ($\times$), and **5b** ($\blacksquare$). Spectra were recorded in 20 mM TRIS buffer (pH 7.3) with 285 mM KCl (total $[\text{Cl}^-] = 300$ mM) at 25 $^{\circ}\text{C}$.

Scheme 1.

Structures of α -helical bundle-forming peptide-dendron hybrids **1** and **2**. Adapted from ref. 15 with permission from the Royal Society of Chemistry.

Scheme 2.

(a) Pattern of hydrophobic (h, black color) and polar (p, cyan color) amino acids employed in the design of α -helical bundle-forming peptide-dendron hybrids **1a** and **2**. (b)

Amphipathic α -helical structure that results from folding of the linear amino acid sequence from (a). Positions i (orange), $i + 7$ (green), and $i + 8$ (yellow) designate the positions to which dendrons have been attached. (c) Side and (d) top views of a parallel four-helix bundle assembled from the designed peptides.

Scheme 3.

Amino acid sequences of peptides **3-5**. Amino acids are identified by single-letter codes: Y = tyrosine; L = leucine; K = lysine; X = propargylglycine.

Scheme 4.
Synthesis of the dendritic azide **11**.

Scheme 5.
Alternative hydrophilic dendrons.

Scheme 6.
Grafting to peptides via CuAAC.