DNA Structures

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Self-Assembly of a Four-Helix Bundle on a DNA Quadruplex**

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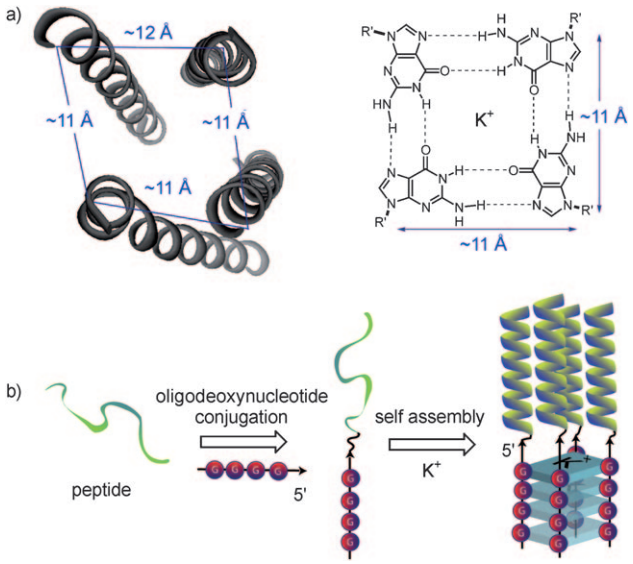
The design of de novo proteins is a prominent goal of chemical biology where functionality often hinges on the specific folded protein structure. A structural motif that is a common focus in the field of protein design is the four-helix bundle,[1] as it is present and crucial to function in several naturally occurring proteins. Natural helix bundle assembly depends on the folding and interhelix contact of protein domains, and de novo proteins have often relied upon synthetic templates to induce specific secondary and tertiary structures.[2,3] The majority of template-assembled synthetic proteins (TASPs),[4] have required covalent tethering of the peptides to one another to control peptide structure and stoichiometry. Several organic templates, including cyclic peptides,[5] cavitands,[6] and porphyrins[7] have been reported in this role. While TASPs have been shown to yield structurally accurate helix bundles, this method of artificial protein assembly has yet to attain full potential in yielding functional constructs.[8–11] A more facile method of assembly would allow for faster screening of larger pools of de novo proteins[12,13] and identification of functional species. An attractive alternative might involve the use of a template that itself assembles through noncovalent forces and abrogates the need for covalent attachment of helices to one another.[14,15]

Higher-order, hydrogen-bonded DNA structures offer a new potential route for the templating of multihelix bundles. The distances between helical chains in a typical, natural four-helix bundle are equivalent to those between substituents projected from hydrogen-bonded guanine, or "G", quartets (as in R′; Scheme 1 a).[16–18] The parallel DNA quadruplex thus offers an intriguing possibility as a scaffold to template the assembly of a four helix bundle protein. The quadruplex, formed intermolecularly from four guanine-rich oligodeoxynucleotide strands, assembles through stacking of three or more G quartets in the presence of potassium counterions. Functionalization of each G-rich strand at one terminus would assemble the four tethered peptides in a parallel, proximal arrangement on one face of the quadruplex (Scheme 1 b).[19] Herein, we report that a random-coil amphiphilic peptide conjugated to the 5′-terminus of the oligodeoxynucleotide 5′-d(TGGGGT)-3′ and, in the presence of potassium ions, self-assembles into a four-helix bundle with a controlled structure, orientation, and stoichiometry.



Scheme 1. a) Distances between coplanar atoms in apoferritin helices (left)[18] and G-quartet (right); b) templation of the four-helix bundle.

A series of conjugates combining peptides with high (**P1**) or low (**P2**) helical propensity with oligodeoxynucleotides capable (**O1**) or incapable (**O2**) of quadruplex formation was prepared by solid-phase synthesis on a 1 μmol scale. As commercial methods of oligonucleotide and peptide syntheses are incompatible, the two polymers were prepared separately and conjugated through an N-hydroxysuccinimide (NHS) ester-modified DNA (see Figure S1 in the Supporting Information). These oligodeoxynucleotides on a resin support, presenting a nine-methylene chain and the NHS-ester-activated functional group at the 5′ terminus, were incubated with a solution of purified peptide (with free N terminus) and *N*,*N*-diisopropylethylamine, then cleaved from the resin with ammonium hydroxide. The resulting conjugates link the peptide to the oligodeoxynucleotide through an amide and nine-methylene chain. All conjugates prepared in this manner were purified by RP-HPLC and verified by MALDI-TOF spectroscopy. Synthetic details are outlined in the supporting information. The biopolymers prepared and analyzed in this study are listed in Table 1. Note that, in **C1**–**3**, the peptide is conjugated at the N terminus, not the C terminus.

Assembly of the DNA quadruplexes was achieved in a buffer solution containing excess K^+ ((250 mM) KCl (250 mM), tris(hydroxymethyl)aminomethane (Tris)/HCl (10 mM), pH 7.5). As in previous TASP studies,[4] circular dichroism (CD) was employed to determine structural characteristics of the assemblies and control reactions.

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Table 1: Peptides,[7] oligodeoxynucleotides, and conjugates prepared.

Biopolymer	Sequence
peptide 1 (**P1**)	AEQLLQEAEQLLQEL
peptide 2 (**P2**)	RPPGFSPFR
oligodeoxynucleotide 1 (**O1**)	d(TG_4T)
oligodeoxynucleotide 2 (**O2**)	d(T_6)
conjugate 1 (**C1**)	**P1**-linker-**O1**
conjugate 2 (**C2**)	**P1**-linker-**O2**
conjugate 3 (**C3**)	**P2**-linker-**O1**

Notably, both parallel quadruplexes and α-helical proteins yielded distinct CD spectra.[20] The assembly of $\mathbf{O1}_4$, the unconjugated quadruplex, led to a spectrum characteristic of a parallel quadruplex, with maxima at 267 and 203 nm, and a minimum at 242 nm (Figure 1a, black). The amphiphilic, unconjugated peptide **P1** gave rise to a spectrum indicating a random-coil structure (Figure 1a, blue). An α-helix protein would normally exhibit minima at 208 and 222 nm. If the parallel DNA quadruplex can template formation of a four-helix bundle, as with $\mathbf{C1}_4$ in potassium ion-containing buffer, a hybrid spectrum of α-helical protein and DNA parallel quadruplex spectra should result. Spectra of the non-quadruplex-forming conjugate **C2**, the quadruplex conjugated to non-helix-forming bradykinin ($\mathbf{C3}_4$), and **C1** in the absence of potassium ions showed no significant α-helical character (see the Supporting Information, Figure S2). However, much more pronounced minima at both 208 and 222 nm, as well as a maximum at 267 and minimum at 242 nm occur in the CD spectrum of $\mathbf{C1}_4$, consistent with the formation of a four-helix bundle quadruplex (Figure 1a, red). This spectrum appears to represent a combination of parallel quadruplex and α-helix peptide signals.

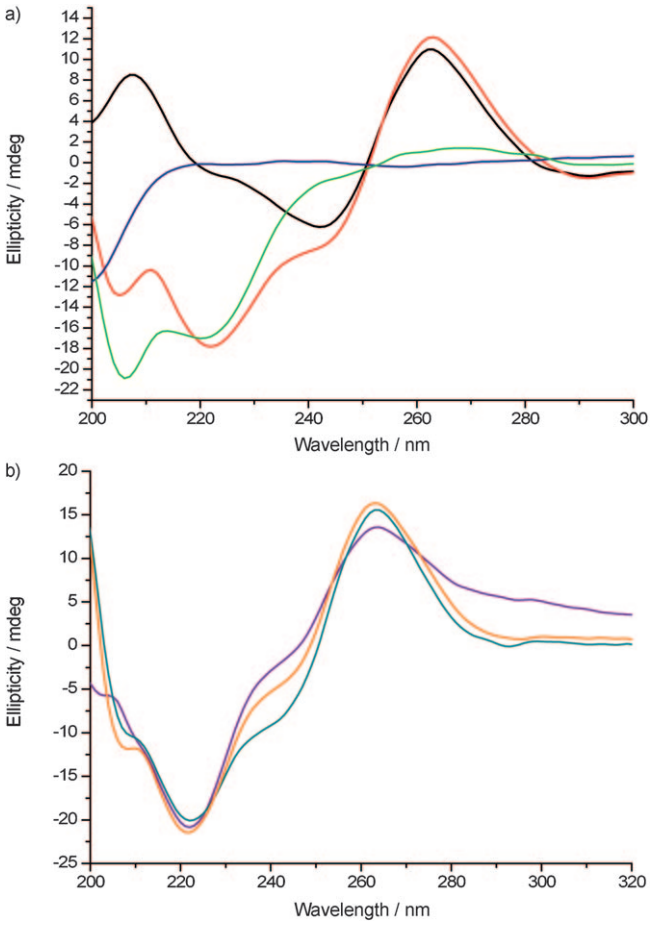

Figure 1. a) CD spectra of **P1** (——), $\mathbf{O1}_4$ (——), $\mathbf{C1}_4$ (——), and $\mathbf{C1}_4$–$\mathbf{O1}_4$ (——); b) CD spectra of $\mathbf{C1}_4$ (——), mixed $\mathbf{O1}_4$ and apoferritin (——), and the added spectra of $\mathbf{O1}_4$ and apoferritin (——). All spectra taken from solution in KCl (250 mM), Tris/HCl (10 mM), pH 7.5 buffer at 25 °C.

The "hybrid" character of the $\mathbf{C1}_4$ spectrum was explored both mathematically and through recreating the signal with a two-component sample. To deconvolute the spectral features of the protein portion of the helix bundle quadruplex assembly, the CD spectrum of unfunctionalized quadruplex $\mathbf{O1}_4$ was subtracted from that of $\mathbf{C1}_4$ (Figure 1a). The resultant spectrum contained only the minima at 208 and 222 nm, characteristic of an α-helix protein (Figure 1a, green). Additionally, by using a controlled two-component system, a CD spectrum similar to that of $\mathbf{C1}_4$ was closely replicated by either adding the CD spectrum of an $\mathbf{O1}_4$ solution to that of apoferritin, a predominantly α-helical protein, or by directly mixing $\mathbf{O1}_4$ and apoferritin (Figure 1b). These spectra overlap almost completely with the spectrum of $\mathbf{C1}_4$.

The binding of 8-anilino-1-naphthalenesulfonic acid (ANS) to proteins has routinely been used to determine their degree of folding. ANS fluoresces more intensely with a blue shift when bound to native or molten globule proteins.[21,22] Addition of ANS (1 μM) to a 50 μM solution of $\mathbf{O1}_4$ gave rise to a slight increase in fluorescence intensity (I_F), possibly owing to interaction with the terminal faces of the quadruplex. ANS at the same concentration, in a solution of 50 μM $\mathbf{C1}_4$, however, exhibited a nearly threefold increase in I_F relative to $\mathbf{O1}_4$ (see the Supporting Information, Figure S3). Neither non-assembled **C1** (conjugate in the absence of K^+), nonconjugated peptide **P1**, nor the non-α-helical $\mathbf{C2}_4$ assembly exhibited a change in I_F upon incubation with ANS. These data support a protein conformational change upon assembly of **C1** strands, and indicate interaction between peptide chains within $\mathbf{C1}_4$.

The fourfold stoichiometry of the $\mathbf{C1}_4$ complex was confirmed by sedimentation equilibrium analyses at three concentrations (3.0, 1.5, and 0.75 μM $\mathbf{C1}_4$ in KCl (250 mM), Tris/HCl (10 mM), pH 7.5) and at three rotor speeds (42, 50, and 60 krpm). Fitted data assuming a single ideal species (using HeteroAnalysis software, UConn) indicated the experimental molecular weight of the complex to be (15019 ± 800) Da, well within the calculated mass of four **C1** monomers, 15404 Da. As this value indicates a tetrameric assembly, analysis within the Monomer–Nmer program (HeteroAnalysis) was conducted with a fixed stoichiometry of $n = 4.00$ ($[\mathbf{C1}_4] = 3.00$ μM, Figure 2; see the Supporting Information, Figure S4 for other concentrations). As a point of comparison, other stoichiometries ($n = 3.00$ and $n = 5.00$) were imposed (see the Supporting Information, Figure S5); among these fits, the residuals for the tetrameric stoichiom-

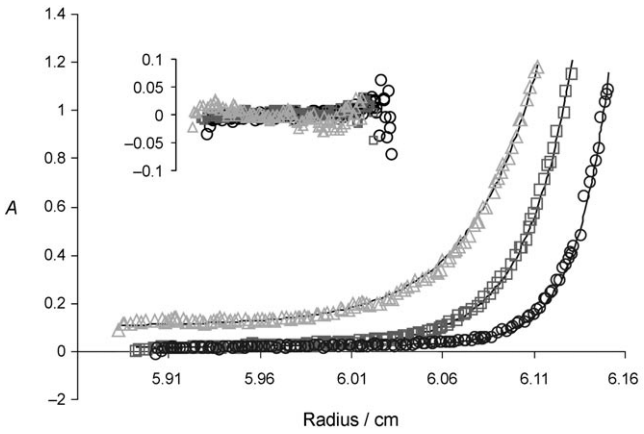

Figure 2. Sedimentation equilibrium analysis of tetrameric $\mathbf{C1}_4$, 3.00 μM at 42 (△), 50 (□), and 60 (○) krpm; inset: residuals. Measurements acquired for absorbance at 260 nm wavelength.

etry were optimal. All calculations and conditions are presented in the Supporting Information.[23,24]

While these data suggest that the stability of the $\mathbf{C1}_4$ assembly is high, in the absence of the counterion, **C1** monomers do not assemble (see the Supporting Information, Figure S2). Stoichiometry was further confirmed by PAGE analyses, as was the ability of the **C1** conjugate to mix with other strands and form asymmetric quadruplex assemblies. Equimolar ratios of **C1** and **O1** strands were mixed in K^+-containing buffer solution and analyzed by nondenaturing PAGE (Figure 3). The statistical mixture of tetrameric

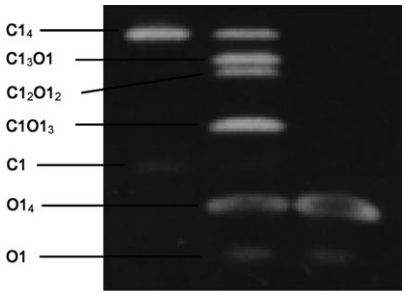

Figure 3. PAGE analysis of mixed quadruplex; lane conditions from left to right are: **C1**, **C1** + **O1**, and **O1**. All samples were incubated with KCl prior to PAGE.

assemblies formed from these two strands should involve five complexes of distinct size: $\mathbf{O1}_4$, $\mathbf{O1}_3\mathbf{C1}$, $\mathbf{O1}_2\mathbf{C1}_2$, $\mathbf{O1C1}_3$, and $\mathbf{C1}_4$.[25] PAGE analysis shows five distinct bands, which fall between the molecular weight reference bands for $\mathbf{C1}_4$ and $\mathbf{O1}_4$. Some monomeric **C1** and **O1** is present, likely as a result of quadruplex dissociation during PAGE. These data support the presence of tetrameric assemblies, and indicate the possibility of forming combinatorial libraries of helix bundles on quadruplex scaffolds.

In summary, we have demonstrated the capability of G-rich DNA to template and control peptide structure and stoichiometry. In future studies we will use a similar approach to assemble helix bundles of different stoichiometries and libraries of de novo proteins to identify functional constructs.

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