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Noncovalent Multivalent Assembly of Jun Peptides on a Leucine Zipper Dendrimer Displaying Fos Peptides

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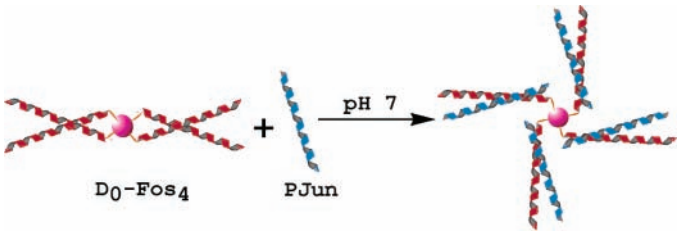
ABSTRACT



The synthesis and characterization of a new leucine-zipper dendrimer (LZD) is reported that displays four copies of the peptide corresponding to the coiled-coiled dimerization domain of Fos. Circular dichroism spectroscopy, fluorescence titration, and sedimentation equilibrium experiments demonstrate that Fos-LZD can noncovalently assemble four copies of the peptide corresponding to the coiled-coil domain of Jun. This work provides the basis for the future construction of noncovalently assembled multivalent protein assemblies displaying any protein of interest.

There is much interest in utilizing noncovalent interactions to control the biomimetic assembly of unimolecular, multimeric, and polymeric structures for biological and nanotechnological applications.[1] Many elegant unimolecular, conformationally unique structures have been recently designed including β-peptides[2] and template-assembled synthetic proteins (TASPs) and other novel foldamers.[3] At the same time more complex polymeric self-assembling structures have also been designed, which include cyclic-peptide nanotubes,[4] peptide-hydrogels,[5] and even PNA-microgels.[6] Dendrimer construction has also witnessed the use of self-assembly utilizing either hydrogen bonding[7] or metal coordination.[8] We have recently combined aspects of peptide self-assembly and dendrimeric architecture in the synthesis of leucine zippers appended to a core dendrimer, providing the necessary framework for constructing multivalent leucine zipper dendrimers (LZDs).[9] These LZDs can noncovalently assemble cognate helical peptides at their periphery. The ability to design biomimetic modular multivalent systems is of much interest in targeting cellular receptors,[10] DNA,[11] and in assembling novel architectures.[1,3]

We have focused on leucine zippers or, more generically, helical coiled-coils[12] as our self-assembly unit as they are very amenable to design[13] and many orthogonal pairs exist

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naturally[14] or can be selected.[15] This potentially allows for the construction of numerous orthogonal self-assembling systems, which is not possible with currently utilized streptavidin−biotin[6,16] or Ni-chelation[17] based multivalent assembly systems. Helical coiled-coils organize their amino acids in a repeating heptad pattern, which when numbered a−g places a specific hydrophobic residue, such as leucine, at the a and d positions to create the knob-hole like hydrophobic interface.

Our previous design strategy utilized electrostatically driven complexation of a coiled-coil tetramer upon the generation-0 PAMAM dendrimeric core at either low or high pH regimes. To expand the utility of our approach toward the facile assembly of discrete multivalent supramolecules at neutral pH, herein we report the synthesis and characterization of a new LZD that is functional at neutral pH. This new LZD displays the coiled-coil domain of the human transcription factor, Fos, and assembles four copies of a peptide corresponding to the coiled-coil domain of its cellular binding partner Jun (Figure 1). Such a system can have great

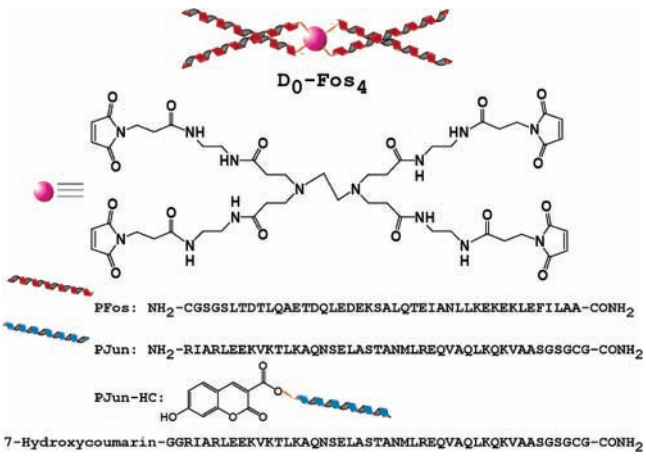

Figure 1. PFos (red) is shown appended to the generation-0 PAMAM dendrimer (purple ball) to result in the LZD D_0-Fos_4. The sequences of PFos (red), PJun (blue), and 7-hydroxycoumarin-appended PJun (PJun-HC) are also shown.

utility in the noncovalent multivalent display of any protein fused to a Jun-coiled-coil. This system is especially attractive as the Fos/Jun coiled-coil pair has been shown to be orthogonal to a multitude of other natural coiled-coil pairs[14] and provides a rational starting point for the future assembly of multiple mutually exclusive LZD/cargo pairs. Multiple designed leucine zippers, as utilized in our previous design, have yet to be systematically tested for orthogonality in competitive cellular environments.

In our design, peptides corresponding to the coiled-coil domains of the well-characterized bZIP protein Fos and Jun[18,19] were chosen as starting points as our self-assembling units. We anticipated that the covalent attachment of four coiled-coils to a dendrimer would result in the undesirable intramolecular stabilization of coiled-coils, which is useful in TASP assemblies.[3] We reasoned that the less stable Fos coiled-coil would be suitable for decorating the core dendrimer on the basis of literature precedence that the Fos/Fos homodimer is less stable than the Jun/Jun homodimer, both of which are an order of magnitude less stable than the Fos/Jun heterodimer.[18,19]

The peptides corresponding to the coiled-coil domains of Fos and Jun, PFos and PJun were synthesized by standard solid-phase Fmoc chemistry on a Rink amide resin. Peptides were cleaved and purified by HPLC and subsequently characterized by amino acid analysis and MALDI mass spectrometry (see Supporting Information). The maleimido-functionalized generation-0 PAMAM dendrimer was reacted chemoselectively with a unique Cys at the N-terminus of the Fos peptide to generate D_0-Fos_4 in 20−25% yield (Supporting Information). We also synthesized the Jun peptide (PJun), with an added 7-hydroxycoumarin (PJun-HC) followed by a Gly-Gly linker at the N-terminus. PJun-HC was synthesized to monitor the noncovalent assembly of PFos/PJun-HC and D_0-Fos_4/4PJun-HC complexes utilizing the Gellman assay,[18] which monitors the relief of excimer-mediated quenching of the PJun-HC homodimer. The respective sequence of the peptides and LZD-dendrimer used in this study are shown in Figure 1.

Our initial studies focused on characterization of D_0-Fos_4 in comparison to PFos. We predicted that D_0-Fos_4 would fold into two coiled-coils (Figure 1) as a disulfide linked Fos-peptide homodimer has been previously been shown to be completely folded.[19] We utilized circular dichroism (CD) to monitor the secondary structure of the dendrimer D_0-Fos_4 in comparison to PFos at pH 7 with 10 mM phosphate, 100 mM NaCl, 1 mM DTT (buffer A). The CD spectra of PFos and the LZD, D_0-Fos_4, were significantly different (Figure 2a) and indicated that PFos is primarily unfolded at 25 °C, whereas the D_0-Fos_4 is 85% folded under the same conditions. The greater helicity of D_0-Fos_4 is likely due to the anticipated covalent stabilization as reported for the disulfide-tethered Fos peptides and also observed in coiled-coil assembly in the seryl tRNA synthetase.[20] To interrogate the stability of D_0-Fos_4 in comparison to PFos, we monitored the temperature-dependent change in secondary structure

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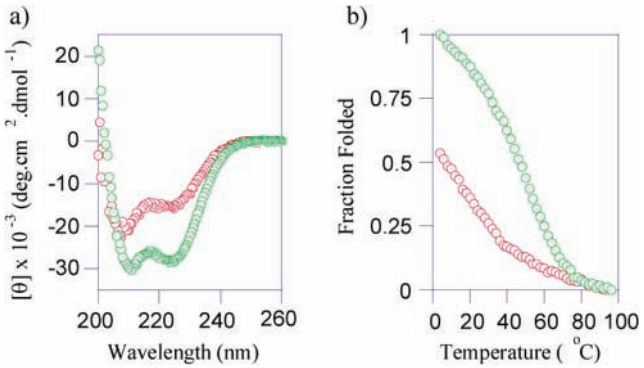

Figure 2. CD secondary structural analysis of PFos and D_0-Fos_4. (a) CD spectra of 20 μM PFos (red) and 5 μM dendrimer D_0-Fos_4 (green) at 25 °C in pH 7.0 buffer containing 10 mM phosphate, 100 mM NaCl, and 1mM DTT (buffer A). (b) Thermal unfolding of 20 μM PFos and 5 μM D_0-Fos_4 in buffer A followed by the change in CD signal at 222 nm.

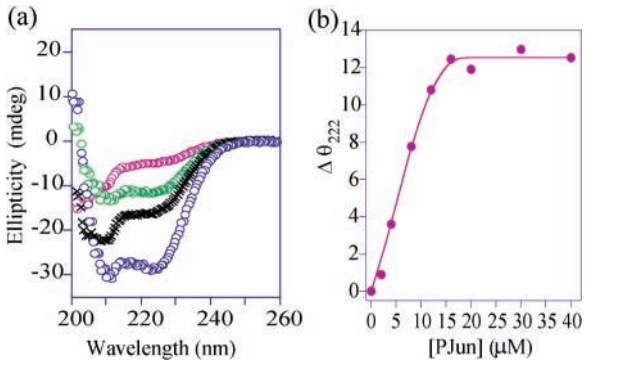

Figure 3. CD analysis of D_0-Fos_4 and PJun complex formation. (a) The uncorrected CD spectra of 2 μM D_0-Fos_4 (green circle), 16 μM PJun (purple circle), and 2 μM D_0-Fos_4 + 16 μM PJun (blue circle). The curve (black cross) represents the calculated weighted average spectra for 2 μM D_0-Fos_4 + 16 μM PJun. (b) Change in PJun helicity upon complexation with Do-Fos_4 at $[\theta]_{222}$ in the presence of 2 μM D_0-Fos_4. Change in θ_{222} of PJun is calculated from $(\theta_{222})_{[D_0-Fos_4+PJun]} - (\theta_{222})_{[PJun]} - (\theta_{222})_{[D_0-Fos_4]}$ All samples were in buffer A.

(Figure 2b) by following the change at $[\theta]_{222}$ by CD spectroscopy.

In the CD melting curve, the apparent T_m (where 50% of the structures are folded) of D_0-Fos_4 at 5 μM (20 μM based on PFos concentration) is ~40 °C higher than that of PFos at 20 μM. The mean residue ellipticity at $[\theta]_{222}$ of D_0-Fos_4 at 4 and 96 °C was taken to represent the theoretical maximum folded and unfolded baselines for D_0-Fos_4 and PFos at these concentrations. Given the relative stability of D_0-Fos_4 we wanted to ensure that it did not form higher order oligomers that would complicate analysis for PJun complexation studies. Toward this end we measured the molecular weight and oligomerization states of the PJun, PJun-HC, and D_0-Fos_4 utilizing sedimentation equilibrium (Supporting Information). Measurements were made on an analytical ultracentrifuge at 20 °C in PBS at three different concentrations. We found that PJun and PJun-HC existed in equilibrium between monomer and dimer, whereas D_0-Fos_4 existed only as a monomer. The dissociation constant of PJun was found to be 45 μM, which was 3.5-fold weaker than that for PJun-HC, which was found to be 13 μM.

Gellman and co-workers have previously reported a similar increase in stability of a Jun-based peptide homodimer upon the incorporation of 7-hydroxycoumarin.[18] Having established that PJun was only partially helical between 0 and 50 μM and that D_0-Fos_4 was completely helical and monomeric, we predicted that it would be possible to monitor the assembly of four copies of peptide Jun upon the LZD D_0-Fos_4 utilizing CD spectroscopy. In our CD experiment, we assumed that the overall helicity of PJun would increase as it was complexed by D_0-Fos_4. To test this, we made a series of samples in pH 7.0 buffer (10 mM phosphate, 100 mM NaCl, 1 mM DTT), such that the concentration of D_0-Fos_4 was constant at 2 μM (8 μM based on PFos concentration), while the concentration of PJun was varied from 0 to 40 μM in the presence and absence of D_0-Fos_4. The results for 2 μM D_0-Fos_4, 16 μM PJun, and 2 μM of D_0-Fos_4 + 16 μM PJun is shown in Figure 3a. It is clear that 2 μM D_0-Fos_4 is fully folded, and 16 μM PJun is primarily unfolded. If there were no interaction between D_0-Fos_4 and PJun, then the experimental spectra of 2 μM D_0-Fos_4 with 16 μM Jun would be equivalent to the sum of the spectra for D_0-Fos_4 (2μM) and PJun (16 μM). However, the CD spectra clearly show that 16 μM PJun + 2 μM of D_0-Fos_4 is considerably more helical than that for the calculated addition spectra. This change in helicity can be directly attributed to change in PJun secondary structure upon complexation with D_0-Fos_4. The result of the complete CD titration experiments is shown in Figure 3b, where the relative change in PJun helicity at 222 nm obtained from the subtraction spectra of $(\theta_{222})_{[D_0-Fos_4+PJun]} - (\theta_{222})_{[PJun]} - (\theta_{222})_{[D_0-Fos_4]}$ is shown as a function of increasing PJun concentration. The titration curve plateaus at 16 μM PJun, suggesting that an 8-fold excess (16 μM) of PJun is required for the complete complexation of 2 μM D_0-Fos_4, where all the PFos tethered to the LZD is completely occupied by PJun. This analysis, although confirming our design strategy, is complicated by the concentration-dependent monomer−dimer equilibrium of PJun.

We further evaluated the complexation of D_0-Fos_4 with PJun utilizing the Gellman assay,[18] where titration of PJun-HC with either D_0-Fos_4 or PFos is expected to lead to an increase in PJun-HC fluorescence due to an increase in the average distance between the 7-hydroxycoumarins attached to dimeric PJun-HC. The change in PJun-HC fluorescence was monitored by excitation at 386 nm and emission at 447 nm. The concentration of PJun-HC was held constant at 1.67 μM, while the concentration of D_0-Fos_4 (0 to 5 μM) or PFos (0 to 20 μM) was varied. The results are shown in Figure 4a, where the change in PJun-HC fluorescence is plotted with respect to the molar ratio of [PFos]/[PJun-HC], ([D_0-Fos_4])/[PJun-HC]. The results from the PJun-HC titration clearly show that both D_0-Fos_4 and PFos can complex PJun-HC, resulting in relief of excimer quenching as the 7-hydroxy-

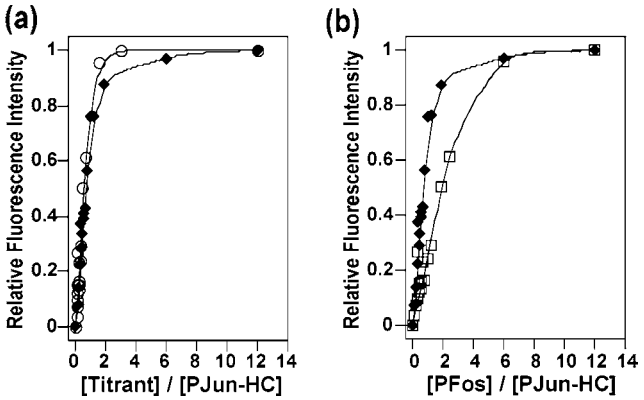

Figure 4. Fluorescence titration of PJun-HC. In (a) the titration of PJun-HC by PFos (♦) and D_0-Fos_4 (○) is shown. In (b) the same data are shown, but D_0-Fos_4 concentration is expressed as [D_0-Fos_4]/4 (□) to compare more directly with PFos (♦) on a per leucine zipper basis. PJun-HC was constant at 1.67 μM; excitation was at 386 nm, and emission recorded at 447 nm.

coumarins are no longer held in close proximity. Moreover, in both cases recovery of fluorescence plateaus when the concentration of PFos is over 6-fold that of PJun-HC. However, whereas PFos was observed to recover 50% of PJun-HC fluorescence at 0.7 molar equiv, D_0-Fos_4 could only recover 50% of PJun-HC fluorescence at 1.9 molar equiv based on a per leucine zipper basis (Figure 4b). This 2.7-fold difference in the ability of D_0-Fos_4 versus PFos to successfully complex PJun-HC can be attributed to the energetic penalty for first having to unfold the covalently stabilized Fos peptides dimers on D_0-Fos_4 prior to complexation with PJun-HC. Alternatively, it is possible that the assembly of four PJun peptides on the D_0-Fos_4 is sterically demanding. It is also interesting to note that the absolute recovered fluorescence of PJun-HC was 25% less when titrated with D_0-Fos_4 relative to PFos, which is very likely due to the four 7-hydroxycoumarins still being held in close proximity when decorated upon the D_0-Fos_4 LZD scaffold (Supporting Information).

Sedimentation equilibrium was utilized to directly interrogate the D_0-Fos_4/PJun complex formation. On the basis of our CD and fluorescence titration experiments we estimated that an 8-fold excess of PJun was required to complex all four sites on D_0-Fos_4. Thus, in the sedimentation equilibrium experiments two different molar ratios were utilized where the D_0-Fos_4/PJun was held at relative ratios of either 1:4 or 1:8. Analysis of the sedimentation equilibrium data indicated that when the molar ratio of D_0-Fos_4/PJun was 1:4, the data was best fit by three species corresponding to D_0-Fos_4/2PJun, PJun-monomer and PJun dimer. However, when the molar ratio of D_0-Fos_4/PJun was 1:8, the data was best fit to three species corresponding to D_0-Fos_4/4PJun, PJun-monomer, and PJun dimer (Supporting Information). Thus, these sedimentation equilibrium results along with the fluorescence and CD experiments indicate that we have successfully assembled four copies of PJun on the D_0-Fos_4 scaffold to afford a noncovalent supramolecular complex of 40,000 Da in molecular weight. The complexationof PJun with D_0-Fos_4 could follow a two-step process, where first two of the PFos peptides on D_0-Fos_4 unfold and bind 2 equiv of PJun, following which the next two Fos peptides unfold and bind another 2 equiv of PJun. Alternatively, the PJun peptides could bind the Fos peptides appended to the dendrimer core one at a time, which does not agree with our sedimentation equilibrium data and would also be energetically unfavorable.

This work demonstrates the noncovalently assembly of four coiled-coil peptides upon a leucine zipper dendrimer-displaying scaffold characterized by CD, fluorescence, and sedimentation equilibrium experiments. These studies also show that the significant covalent stabilization of leucine zippers by the dendrimeric scaffold has to be overcome for the successful noncovalent assembly of cognate peptides. Future work in this area will entail the expression of proteins directly attached to leucine zippers, such as PJun, such that they can be complexed upon LZDs, such as D_0-Fos_4, to instantly afford functional multivalent protein complexes. The orthogonality of different leucine-zipper pairs can potentially allow for the coexistence of many such assemblies that can be utilized for DNA binding,[11] targeting receptors on cell-surfaces,[10] or in mimicking multivalent enzymatic complexes.[21]

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Supporting Information Available: Synthesis, characterization, fluorescence and sedimentation equilibrium data for D_0-Fos_4, PFos, PJun, and PJun-HC. This material is available free of charge via the Internet at http://pubs.acs.org.

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