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# A Reductive Trigger for Peptide Self-Assembly and Hydrogelation

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***Abstract:*** Stimulus-responsive peptide self-assembly provides a powerful method for controlling self-assembly as a function of environment. The development of a reductive trigger for peptide self-assembly and subsequent hydrogelation is described herein. A self-assembling peptide sequence, Ac-C(FKFE)$_2$CG-NH$_2$, was cyclized via disulfide bonding of the flanking cysteine residues. The macrocyclic form of this peptide enforces a conformational restraint that prevents adoption of the $\beta$-sheet conformation that is required for self-assembly. Upon reduction of this disulfide bond, the peptide relaxes into the preferred $\beta$-sheet conformation, and immediate self-assembly into fibrillar superstructures occurs. At sufficient peptide concentration, self-assembly is accompanied by the formation of rigid, viscoelastic hydrogels.

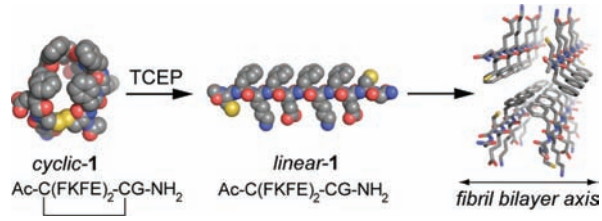
Noncovalent peptide self-assembly has been exploited for the preparation of dynamic and functional hydrogel nanomaterials that have been applied to regenerative medicine, wound healing, drug delivery, and biosensing.[1−5] Upon undergoing self-assembly at sufficient concentration, peptide-based fibrils form entangled networks that reduce the flow of solvent, resulting in the hydrogel state.

Stimulus-responsive peptide self-assembly provides a powerful method for controlling hydrogelation as a function of environment.[6] Peptides have been designed to undergo self-assembly in response to environmental and chemical stimuli,[4] including pH,[7] ionic strength,[8] temperature,[9] light,[10] and enzymatic manipulation.[11] Of these stimuli-responsive systems, only ones based on pH or enzyme-triggered self-assembly are highly responsive to biological microenvironments. For example, self-assembly can be triggered within a cell by choosing an enzyme activator that is not found in the extracellular environment. The development of additional microenvironment-sensitive triggers for self-assembly will enable more diverse biological application of peptide-derived self-assembled materials.

Imposing a conformational constraint that prevents adoption of the $\beta$-sheet secondary structure required for the self-assembly of short peptides is a possible strategy for controlling peptide self-assembly. We postulated that flanking a short self-assembling peptide sequence with cysteine (Cys) residues would enable the macrocyclization of these peptides, preventing $\beta$-sheet formation and self-assembly in the cyclic form (Scheme 1). This constraint could be removed by simple reduction of the disulfide bond, resulting in relaxation to the stable $\beta$-strand and thus triggering self-assembly. A reductive trigger could facilitate more nuanced stimulus-responsive self-assembly in a biological milieu. For example, a peptide could remain monomeric in the extracellular oxidizing environment but assemble in the reducing environment of the cell or in the vicinity of tumors, which often have local extracellular reducing environments.[12]

The amphipathic Ac-(FKFE)$_2$-NH$_2$ sequence, which has a high propensity to undergo self-assembly into water-soluble amyloid-like fibrillar bilayers, was chosen as a model sequence to explore the concept of a reductive trigger for peptide self-assembly.[13,14] This sequence was flanked with Cys residues, and a Gly was included at the C-terminus to avoid problems with Cys racemization during resin loading; this gave the target sequence, Ac-C(FKFE)$_2$CG-NH$_2$ (*linear*-**1**, Scheme 1). *Linear*-**1** was synthesized by standard Fmoc solid-phase peptide synthesis and then oxidatively cyclized by diluting *linear*-**1** ($\leq$100 $\mu$M, acetonitrile/water) and incubating it with 4,4′-pyridyldisulfide (4-PDS) for 24 h, giving *cyclic*-**1**.[15] The presence of high ratios of acetonitrile prevented self-assembly of *linear*-**1**, thus facilitating these operations. HPLC analysis indicated nearly quantitative conversion of *linear*-**1** to *cyclic*-**1** under these conditions.

***Scheme 1.*** Cyclic to Linear Peptide Conformational Switch Using a Reductive Trigger



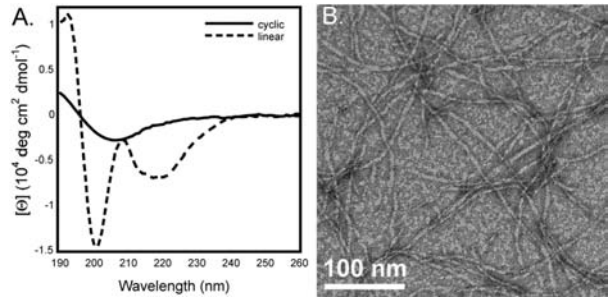




***Figure 1.*** (A) CD spectra of *cyclic*-**1** (1 mM in water) and *cyclic*-**1** + TCEP (giving *linear*-**1**; 1 mM peptide, 10 mM TCEP). (B) Negatively stained TEM image of fibrils derived from *linear*-**1** (*cyclic*-**1** + TCEP).

The self-assembly behavior of the *cyclic*-**1** peptide was then assessed. Self-assembly studies were performed by dissolving the peptides in unbuffered water [pH 2.4−3.0 because of the presence of residual trifluoroacetic acid (TFA) from HPLC purification]. Circular dichroism (CD) spectra of *cyclic*-**1** at concentrations ranging from 100 $\mu$M to 10 mM showed a characteristic minimum at 208 nm indicative of a random-coil structure (Figure 1A and Figure S2 in Supporting Information). Negatively stained transmission electron microscopy (TEM) images of solutions of *cyclic*-**1** showed no evidence of fibrillar self-assembly. Solutions of *cyclic*-**1** were stable for months, with no apparent self-assembly even after extended incubation at all concentrations studied (Figure S9).

The addition of reductant to *cyclic*-**1** resulted in immediate disulfide bond reduction and self-assembly of the resulting *linear*-**1**



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peptide. Tris(2-carboxyethyl)phosphine (TCEP) was initially chosen as the reductant, and 1.2−10 molar equiv relative to disulfide was added. Dithiothreitol (DTT) was also an effective reductant, triggering formation of *linear*-**1** and self-assembly at the same concentrations as TCEP. The CD spectrum of *cyclic*-**1** + TCEP displays two major minima at 217 and 200 nm (Figure 1A). The minimum at 217 nm is classically indicative of the $\beta$-sheet structure required for peptide self-assembly of the $(FKFE)_2$ sequence. The minimum at 200 nm has been observed previously with the $(FKFE)_2$ peptide and attributed to $\pi-\pi^*$ effects resulting from aromatic $\pi-\pi$ interactions in the hydrophobic interior of the self-assembled bilayer structure.[14,16] Negatively stained TEM images of *linear*-**1** solutions displayed abundant peptide fibrils that were 3.5 ± 0.4 nm wide (Figure 1B). Self-assembly was observed at all concentrations studied (100 $\mu$M to 10 mM; see Figures S3−S8 for CD spectra and TEM images). This data indicates rapid peptide self-assembly upon disulfide bond reduction and relaxation of conformational constraint.

Hydrogelation was observed in a concentration-dependent manner upon addition of reductant to solutions of *cyclic*-**1**. At low concentrations (≤1 mM), *linear*-**1** solutions did not form hydrogels. At a peptide concentration of 10 mM (∼0.9 wt %), addition of TCEP resulted in the immediate formation of self-supporting hydrogels, as indicated by the vial inversion test (Figure 2A). The viscoelastic properties of these hydrogels were characterized rheologically: the storage modulus ($G'$) and loss modulus ($G''$) indicate the elasticity and viscosity of the hydrogel, respectively (Figure 2B). A rigid hydrogel is defined in practice as having a $G'$ value that exceeds the $G''$ value by an order of magnitude.[17] Immediately after addition of reductant, *linear*-**1**-derived hydrogels exhibited $G' = 79.9 \pm 1.3$ Pa and $G'' = 7.9 \pm 0.8$ Pa. The resulting hydrogels were stable at oscillatory frequencies of up to 30 rad $s^{-1}$.

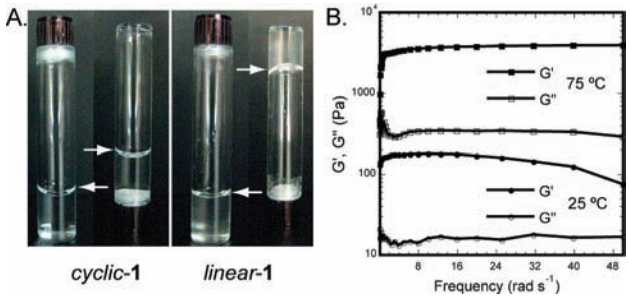


***Figure 2.*** (A) Images of vial inversion tests for (left) *cyclic*-**1** (vial upright and inverted, white arrows indicate the liquid levels) and (right) *linear*-**1** (i.e., *cyclic*-**1** + TCEP; vial upright and inverted). (B) Rheological storage moduli ($G'$) and loss moduli ($G''$) of hydrogels derived from *linear*-**1** (i.e., *cyclic*-**1** + TCEP) at 25 and 75 °C.

Rigidification of the hydrogel was observed when the gel was allowed to mature for 24 h. The $G'$ and $G''$ values increased to 160.2 ± 14.5 and 15.8 ± 1.3 Pa, respectively, and these gels were stable to 50 rad $s^{-1}$. This is most likely due to the formation of covalent disulfide cross-links between fibrils (or between $\beta$-strands within a $\beta$-sheet or between $\beta$-sheets in the bilayer) as reductant was consumed and an oxidizing environment subsequently established.[18,19] Disulfide bond formation was indicated by determining the free thiol concentration using Ellman's reagent.[15] The thiol concentration was 8.2 mM immediately after addition of TCEP reductant. After 24 h, this concentration was reduced to 2.3 mM, consistent with thiol oxidation leading to hydrogel stabilization and rigidification. TEM images of the freshly prepared gel and the gel after maturation for 24 h (Figure S12) showed only subtle differences in fibril density, suggesting that intrasheet or intersheet bilayer disulfide bonds dominate. Increasing the temperature also resulted in rigidification of the hydrogels, as has been observed in other systems (at 75 °C, $G' = 3386.9 \pm 87.4$ Pa and $G'' = 326.2 \pm 15.6$ Pa; see the Supporting Information for temperature-sweep data).[9]

This work has demonstrated that introduction of a conformational constraint by disulfide-bond-mediated peptide cyclization is a viable strategy for controlling peptide self-assembly. This strategy should be generally applicable to many short self-assembling peptide sequences. Significantly, reducing and oxidizing conditions are important in biologically relevant microenvironments, and the development of a reductive trigger for stimulus-responsive self-assembly has considerable potential for application in biotechnology. Exploration of potential applications is currently underway in our lab. Finally, use of conformational constraints to control peptide self-assembly need not be limited to reductive triggers. Many reversible chemical bonds could potentially be utilized to the same effect by relying on other environmental stimuli to promote self-assembly, and the realization of this potential will open novel and imaginative opportunities for the exploitation of peptide self-assembly in biomaterials and biomedical research.

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**Supporting Information Available:** Experimental details and additional CD spectroscopic data and TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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