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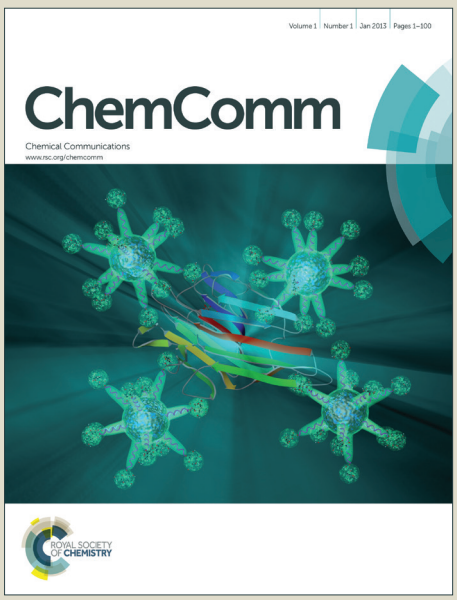

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ARTICLE TYPE

Proteolytic Stability of Amphipathic Peptide Hydrogels Composed of Self-Assembled Pleated *β*-Sheet or Coassembled Rippled *β*-Sheet Fibrils

Ria J. Swanekamp, Jade J. Welch and Bradley L. Nilsson*

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Hydrogel networks composed of rippled *β*-sheet fibrils of coassembled D- and L-Ac-(FKFE)$_2$-NH$_2$ amphipathic peptides exhibit proteolytic stability and increased rheological strength compared to networks of self-assembled L-Ac-(FKFE)$_2$-NH$_2$ pleated *β*-sheet fibrils. Modifying the ratios of L and D peptides in the coassembled rippled *β*-sheet fibrils alters the degradation profiles of these hydrogel networks.

Self-assembled peptide nanofibrils composed of amphiphilic, *α*-helical, and *β*-sheet peptides have been exploited in bottom-up approaches to create novel bionanomaterials.[1-3] These materials include hydrogels for *ex vivo* tissue engineering, wound healing, and drug delivery.[4-6] Amphipathic *β*-sheet peptides with alternating polar and nonpolar residues are a privileged class of self-assembling peptide that have found wide use as noncovalent hydrogel biomaterials.[3] Amphipathic peptides of this class readily assemble into bilayer nanoribbons with a hydrophobic core and a hydrophilic exterior face (Figure 1A) that is solvent exposed, providing water solubility and facilitating formation of entangled hydrogel networks.[7-8] These self-assembled hydrogels have been engineered to be responsive to environmental stimuli to facilitate triggered assembly, disassembly, or degradation. Hydrogels with novel emergent mechanical and degradation properties will enable next-generation applications as functional biomaterials.

We recently reported a novel class of peptide-based nanofibril composed of equimolar mixtures of enantiomeric amphipathic peptides that coassemble into "rippled *β*–sheet" structures as predicted by Pauling.[9] We found that D and L-Ac-(FKFE)$_2$-NH$_2$ peptides coassemble into *β*–sheets with alternating D and L-sequences to form rippled *β*–sheets (Figure 1B) that are distinguishable from self-assembled, single enantiomer, pleated *β*-sheet fibrils (Figure 1A).[9-10] It was found that coassembly of enantiomeric peptides into rippled *β*–sheets was enthalpically favorable, presumably due to the altered packing orientation of rippled *β*–sheets relative to pleated *β*–sheets. Schneider *et al*. have also recently reported that coassembly of enantiomeric (VKVK)$_n$ hairpin peptides provides hydrogels with enhanced mechanical rigidity relative to the single enantiomer self-assembled fibrils; their data suggests that rippled *β*-sheet formation occurs with this peptide sequence as well.[11] Wetzel *et al*. have also demonstrated rippled *β*-sheet formation with enantiomeric poly-Gln amyloid peptides.[12] Herein, we report on the emergent rheological and degradation properties of hydrogels

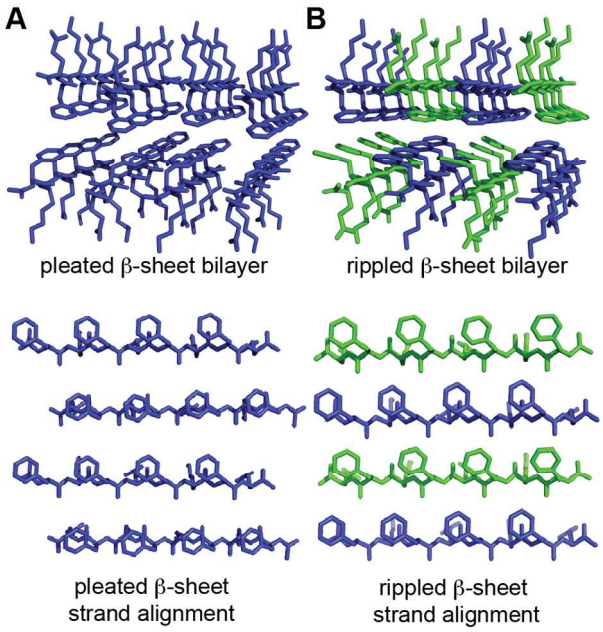

Fig. 1 Schematic representation of assembled *β*–sheets fibrils composed of amphipathic Ac-(FKFE)$_2$-NH$_2$ peptides. A. Pleated *β*–sheet fibrils composed of L-Ac-(FKFE)$_2$-NH$_2$; at the top is shown the *β*–sheet bilayer structure and the out-of-register *β*–sheet packing is portrayed on the bottom. B. Rippled *β*–sheet fibrils composed of coassembled enantiomeric D- and L-Ac-(FKFE)$_2$-NH$_2$ peptides (D shown in green).

derived from rippled *β*–sheets of D and L-Ac-(FKFE)$_2$-NH$_2$ peptides. Specifically, we have found that rippled *β*–sheet hydrogels of coassembled D and L-Ac-(FKFE)$_2$-NH$_2$ have moderately enhanced rigidities relative to the corresponding pleated *β*-sheet materials and that rippled *β*–sheets are stable to proteolytic degradation. Pleated/rippled sheet hybrid fibrils composed of varying ratios of D- and L-peptide exhibit modified hydrogel degradation profiles, demonstrating the tunability of noncovalent amphipathic peptide hydrogel clearance rates.

Based on the proposed use of amphipathic peptide nanofibrils for *in vivo* applications, we sought to characterize the proteolytic degradation profiles of rippled *β*-sheet nanofibrils relative to single enantiomer pleated *β*-sheet counterparts. It has been previously demonstrated that self-assembled pleated sheet fibrils composed of natural L-enantiomeric peptides can be

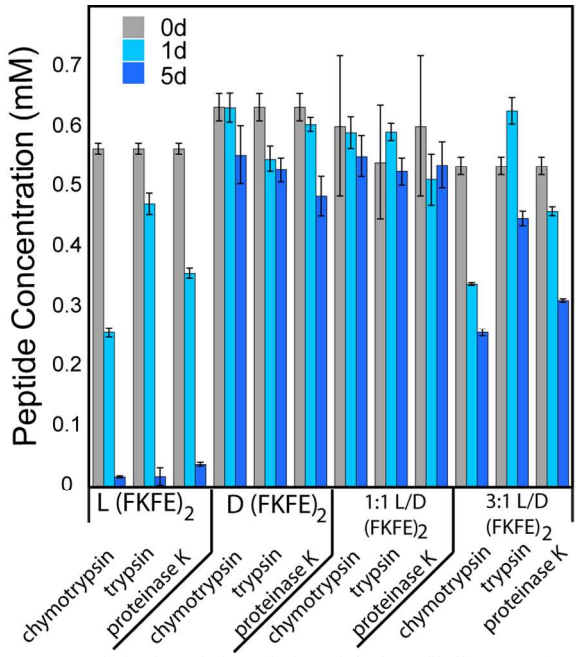

Fig. 2 Proteolytic degradation of pleated β–sheet fibrils (L-Ac-(FKFE)$_2$-NH$_2$ and D-Ac-(FKFE)$_2$-NH$_2$ fibrils), rippled β–sheet fibrils (1:1 L/D-Ac-(FKFE)$_2$-NH$_2$ fibrils), and hybrid pleated/rippled β–sheet fibrils (3:1 L/D-Ac-(FKFE)$_2$-NH$_2$ fibrils) by chymotrypsin, trypsin, and proteinase K over five days.

proteolytically degraded.[13] Indeed, fibrils have been designed with specific protease cleavage sequences to facilitate selective clearance in response to disease-relevant proteases.[14-15] As would be expected, pleated fibrils composed of D-amphipathic peptides are resistant to protease degradation.[16-17] To date, there have been no reports of the proteolytic degradation of rippled β-sheet fibrils composed of coassembled L-and D-amphipathic peptides. We hypothesized that rippled β-sheet fibrils would be resistant to protease degradation. However, peptide self-assembly is an equilibrium process that, at endpoint, exists as a mixture of fibril and monomer which associates/dissociates from the fibril ends.[18] For many peptide fibrils, the equilibrium overwhelmingly favors fibrils, but the possibility of protease degradation of rippled β-sheet fibrils over time as small amounts of L-monomer dissociate from the fibrils must be considered.

We first examined the proteolytic degradation of self-assembled L-Ac-(FKFE)$_2$-NH$_2$ peptide fibrils compared to coassembled D and L-Ac-(FKFE)$_2$-NH$_2$ peptide fibrils. Proteolytic degradation of single enantiomer pleated β-sheet fibrils (L-Ac-(FKFE)$_2$-NH$_2$ and D-Ac-(FKFE)$_2$-NH$_2$ fibrils) and coassembled rippled β-sheet fibrils (1:1 L-/D-Ac-(FKFE)$_2$-NH$_2$ and 3:1 L-/D-Ac-(FKFE)$_2$-NH$_2$) by α-chymotrypsin, trypsin, and proteinase K were assessed. Fibrils were prepared by dissolving purified peptides in water (~0.5 mM total peptide, see ESI for details). These fibrils were then exposed to 1 mg mL^{-1} of each protease and degradation was monitored by removing aliquots of these mixtures, disaggregating the fibrils by dilution into DMSO, and determining the concentration of intact peptide by immediate injection onto an HPLC column and correlation of integrated peak area to a standard curve (see ESI for details).

We found that pleated β-sheet and rippled β-sheet fibrils exhibited varying susceptibilities to protease degradation (Figure 2). After 24 h, L-Ac-(FKFE)$_2$-NH$_2$ fibrils showed significant degradation with all three proteases with nearly half the peptide consumed. After five days, less than five percent of the initial 0.5 mM L-Ac-(FKFE)$_2$-NH$_2$ peptide remained (17 ± 2 μM for chymotrypsin, 17 μM ± 3 μM for trypsin, and 38 ± 15 μM for proteinase K). In contrast, pleated β-sheet fibrils of D-Ac-(FKFE)$_2$-NH$_2$ were only minimally degraded over five days (555 ± 49 μM for chymotrypsin, 530 μM ± 33 μM for trypsin, and 486 ± 21 μM for proteinase K). Significantly, 1:1 coassembled L/D-Ac-(FKFE)$_2$-NH$_2$ rippled β-sheet fibrils also resisted degradation, indicating that rippled sheet packing offers protection to protease-catalyzed hydrolysis for the susceptible L-peptides (553 ± 35 μM for chymotrypsin, 527 μM ± 23 μM for trypsin, and 538 ± 39 μM for proteinase K). This also indicates that dissociation of peptides from these assembled fibrils occurs slowly.

The 3:1 L/D-Ac-(FKFE)$_2$-NH$_2$ coassembled fibrils were degraded by proteases, albeit at a much slower rate and to a lesser extent that the pleated sheet L-Ac-(FKFE)$_2$-NH$_2$ fibrils. After five days, the overall peptide concentration in these 3:1 coassembled fibrils was reduced by approximately one half (258 ± 6 μM peptide remained with chymotrypsin, 313 ± 3 μM with trypsin, and 449 ± 12 μM with proteinase K). This result is consistent with the previous findings in that, at a 3:1 ratio of L to D peptide, it is expected that the resulting fibrils are hybrids of pleated and rippled β-sheet structures. A reduction of the total peptide by half is consistent with nearly complete degradation of the pleated β-sheet segments of these fibrils (composed of L-Ac-(FKFE)$_2$-NH$_2$); the rippled β-sheet segments are resistant to degradation as demonstrated by the 1:1 L to D coassembled materials. This indicates that altering the ratio of L and D peptides in hybrid pleated/rippled β-sheet fibrils can facilitate tunable proteolytic degradation of the overall peptide structure.

At high concentrations, fibrils composed of mixed amphipathic β-sheet peptides can form entangled hydrogel matrices that have been exploited as functional biomaterials.[11, 19-20] Based on the aforementioned studies, we hypothesized that rippled β-sheet fibrils would form hydrogels that resist degradation by proteases and conducted studies to test this hypothesis. Ac-(FKFE)$_2$-NH$_2$ fibrils have been shown to form hydrogels at concentrations above 6 mM.[21-22] Hydrogels of L-Ac-(FKFE)$_2$-NH$_2$ pleated sheet fibrils, D-Ac-(FKFE)$_2$-NH$_2$ pleated sheet fibrils, and 1:1 L- /D-Ac-(FKFE)$_2$-NH$_2$ rippled sheet fibrils were prepared by dissolving these peptides at 8 mM total peptide in water with 1 mg mL^{-1} chymotrypsin (Figure S1). All three fibril types formed optically transparent, stable gels under these conditions. Under these conditions, significant creep of the L-Ac-(FKFE)$_2$-NH$_2$ pleated sheet hydrogel was observed after ~4 h, indicating a degradation of the hydrogel fibril network. After longer incubation times, the hydrogel integrity was nearly completely compromised. In contrast, the D-Ac-(FKFE)$_2$-NH$_2$ pleated sheet hydrogel and the 1:1 L-/D-Ac-(FKFE)$_2$-NH$_2$ rippled sheet hydrogel maintained their integrity after 4 h. Even after several days of incubation, these hydrogels showed only minimal loss of hydrogel integrity.

We further characterized proteolytic degradation of pleated and rippled β-sheet hydrogel networks using oscillatory rheology. Quantification of hydrogel viscoelastic properties were conducted by measuring the storage modulus (G′) and loss modulus (G″) of

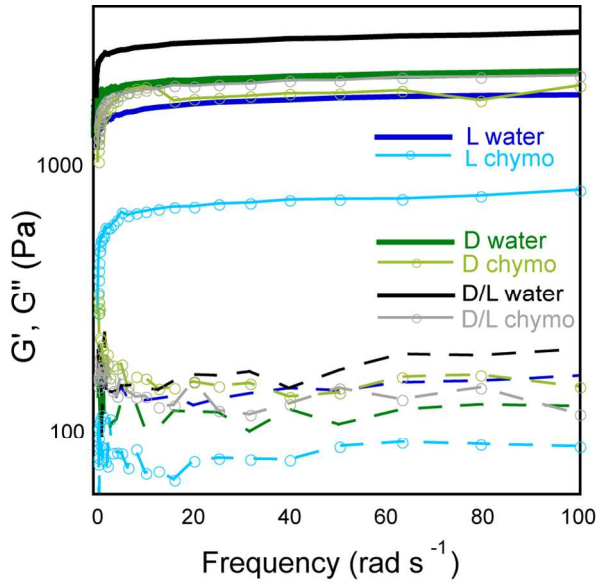

Fig. 3 Rheological dynamic frequency sweep with and without chymotrypsin present for L-Ac-$(FKFE)_2$-NH_2 hydrogels, D-Ac-$(FKFE)_2$-NH_2 hydrogels, and 1:1 L-/D-Ac-$(FKFE)_2$-NH_2 rippled sheet hydrogels. Total peptide concentration was 8 mM and chymotrypsin concentration was 0.5 mg mL^{-1}. G′ values are solid lines and G″ values are dashed lines.

L-Ac-$(FKFE)_2$-NH_2 pleated sheet hydrogels, D-Ac-$(FKFE)_2$-NH_2 pleated sheet hydrogels, and 1:1 L-Ac-$(FKFE)_2$-NH_2/D-Ac-$(FKFE)_2$-NH_2 rippled sheet hydrogels (8 mM total peptide) using rheological dynamic frequency sweep experiments conducted in the linear viscoelastic region (Figure 3). Schneider and coworkers have reported that hydrogels composed of equimolar mixtures of enantiomeric $(VK)_4V^DPPT(KV)_4$-NH_2 (MAX1) β-hairpin peptides (presumably assembled into rippled β-sheet fibril networks) exhibit enhanced material rigidity relative to hydrogels composed of either single enantiomer alone (L or D).[11] We found that rippled sheet hydrogels of Ac-$(FKFE)_2$-NH_2 also exhibited enhanced rigidity relative to pleated sheet hydrogels. Frequency sweep experiments were conducted 2 h after peptide dissolution in water. We found that pleated sheet hydrogels of L-Ac-$(FKFE)_2$-NH_2 and D-Ac-$(FKFE)_2$-NH_2 had G′ values of 1855 ± 138 Pa and 1993 ± 175 Pa, respectively (Figure 3). Rippled sheet hydrogels of 1:1 L-Ac-$(FKFE)_2$-NH_2/D-Ac-$(FKFE)_2$-NH_2 had a higher G′ value of 2674 ± 536 Pa (Figure 3).

We subsequently conducted frequency sweep experiments of each of these hydrogels with 0.5 mg mL^{-1} chymotrypsin; the hydrogels were allowed to stand in the presence of protease for 2 h before rheological analysis. In the presence of chymotrypsin, the L-Ac-$(FKFE)_2$-NH_2 hydrogel showed a significant loss in G′ and G″ (628 ± 215 Pa and 81 ± 4 Pa, respectively) relative to the hydrogel without protease. Incubations times longer than 2 h provided storage and loss moduli values that were consistent with complete loss of hydrogel integrity. The D-Ac-$(FKFE)_2$-NH_2 pleated sheet hydrogel showed no loss in gel rigidity in the presence of chymotrypsin. The 1:1 L-/D-Ac-$(FKFE)_2$-NH_2 rippled sheet hydrogel displayed only a slight loss in rigidity (G′ decreased to 1960 ± 425 Pa); this loss in rigidity is presumably due to imprecision in determining the relative concentrations of L to D peptide in these experiments. Thus, rippled β-sheet hydrogels resist degradation by proteases.

Herein, we have demonstrated that rippled β-sheet materials composed of coassembled enantiomeric amphipathic peptides are resistant to proteolytic degradation. In contrast, pleated β-sheet fibrils of self-assembled L-peptides are readily degraded by common proteases. Hybrid rippled/pleated sheet materials created by altering the ratio of L to D peptide incorporated into the fibril exhibit tunable degradation profiles. These properties are attractive for applications in biological environments in which the ability to alter degradation profiles of these materials is desired. These findings facilitate the creation of next-generation materials composed of self- or coassembled amphipathic peptides for biological applications including *ex vivo* tissue culture, wound healing, and immunology.

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Notes and references

Department of Chemistry, University of Rochester, Rochester, NY, 14627-0216, USA. E-mail: nilsson@chem.rochester.edu; Fax: +1 585 276-0205; Tel: +1 585 276-3053

† Electronic supplementary information (ESI) available: Detailed experimental procedures, peptide purification and characterization data. See DOI: 10.1039/b000000x/

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