Investigating the Effects of Aromatic Amino Acids on Amphipathic Peptide Self-Assembly and Emergent Hydrogel Viscoelasticity

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Introduction

Self-assembled peptides have been exploited to create novel biomaterials, including hydrogels for tissue engineering, wound-healing and drug delivery [1-3]. Amphipathic β -sheet peptides composed of alternating hydrophobic and hydrophilic amino acids (with general sequence (XZXZ)_n where X is nonpolar and Z is polar) are a privileged class of self-assembling peptide that have been widely used in the design of hydrogel materials [4]. The (FKFE)₂ peptide is a prominent member of this class of material that has been frequently studied [5,6]. This peptide self-assembles into putative β -sheet bilayer nanoribbons; molecular dynamics studies suggest that the alignment of peptides within the β -sheet structure requires the *N*-terminal Phe residue to “dangle” out of register (Figure 1) [6]. We have adopted this peptide specifically to understand the impact of general hydrophobic and more specific aromatic π - π effects on peptide self-assembly [7,8]. Our studies have shown that substitution of the Phe residues in (FKFE)₂ peptides with either aromatic or nonaromatic residues has an impact on both self-assembly propensity and on the morphology of the self-assembled materials. Self-assembly propensity can be more directly correlated to the hydrophobicity of nonpolar amino acids rather than to the aromatic character of these residues.

While these studies suggest that self-assembly propensity for this class of peptides is correlated to hydrophobicity of the nonpolar amino acids, the emergent hydrogel properties of networks of these fibrils appear to be correlated with the aromatic character of the nonpolar residues in the constituent peptides [8]. Specifically, it was found that the elasticity of hydrogels derived from Ac-(XKXX)₂-NH₂ peptides in which X was Val, Ile, Phe, pentafluoro-phenylalanine (F₅-Phe or F⁵F), or cyclohexylalanine (Cha) depended on the aromaticity of the X residue. Hydrogels of the Ac-(ChaKChaK)₂-NH₂ and Ac-(IKIK)₂-NH₂ peptides (G' values of ~75 and 230 Pa respectively) were significantly weaker than those of Ac-(FKFK)₂-NH₂ and Ac-(F⁵FKF⁵FK)₂-NH₂ (G' values of ~1640 and 1960 Pa respectively). It is not immediately apparent why this trend is observed. If hydrogel rigidity is a function of fibril density

Fig. 1. Structural representation of Ac-(FKFE)₂-NH₂ β -sheet bilayer fibrils. The β -sheet axis is perpendicular to (extending from and descending into) the page. The *N*-terminal Phe residue, which is proposed to hang out-of-register, is shown in green.

alone, then the viscoelasticity of each of these hydrogels should be similar, since the degree of fibrillization for each peptide was shown to be similar [8]. These observations suggest that specific aromatic effects may influence formation of the fibril hydrogel network. Based on the proposed structure (Figure 1) that indicates β -sheet alignment with the *N*-terminal residue of the constituent peptides out of register, we postulated that specific cross-fibril interactions involving this *N*-terminal residue may occur during hydrogelation, accounting for the observed differences in elasticity between aromatic versus nonaromatic hydrogels. Our data suggests that these hypothetical interactions are apparently more favorable if aromatic residues are present at

the *N*-terminus. If this is true, then modification of only the *N*-terminal residue of these peptides could change the emergent viscoelasticity of the resulting hydrogels. For example, modifying Ac-(ChaKXChaK)₂-NH₂ (which has a reported *G'* value of 75 Pa) to Ac-FK(ChaK)₃-NH₂ would be expected to dramatically increase the elasticity of the resulting hydrogel. Herein, we report the results of an initial assessment of this hypothesis.

Results and Discussion

In order to assess our hypothesis, the peptides shown in Table 1 were designed and synthesized. The peptides were based on the aromatic Ac-(^{F5}FK^{F5}FK)₂-NH₂ or the nonaromatic Ac-(ChaKChaK)₂-NH₂ core sequences with modifications made only to the *N*-terminal nonpolar residue. Based on our central hypothesis, we predicted that changing the *N*-terminal residue of the rigid Ac-(^{F5}FK^{F5}FK)₂-NH₂ hydrogels to a nonaromatic amino acid should weaken the gels. Conversely, changing the *N*-terminal residue of the weak Ac-(ChaKChaK)₂-NH₂ hydrogels to an aromatic residue should strengthen the resulting gel.

Peptides were synthesized by standard solid phase peptide methods. The peptides were purified by high pressure liquid chromatography (HPLC) and characterized by MALDI-TOF mass spectroscopy. Peptide concentrations were determined by correlation to HPLC standard curves for each peptide that were calibrated by amino acid analysis [8]. Peptide self-assembly was initiated by dissolution in 200 mM NaCl to obtain samples with final peptide concentrations of 8 mM. TEM imaging confirmed assembly into fibrils (see Figure 2 for representative images). (Note that these cationic peptides do not spontaneously self-assemble without NaCl, which acts to shield repulsive charge effects; 200 mM NaCl was sufficient to promote assembly of each of the peptides studied herein). The samples were then vortexed (1 min) and sonicated (5 min). The cycle was repeated twice to obtain a homogenous mixture that was then stored at room temperature for 1 day to allow homogenous hydrogels to form. Hydrogel viscoelasticity was characterized by oscillatory rheology (TA instruments AR-G2 rheometer) to determine to storage (*G'*) and loss (*G''*) moduli. Frequency sweep experiments were performed on gels (150 μL) that were transferred onto the rheology plate using a capillary piston pipettor specifically manufactured to handle viscous, soft materials. Experiments were performed using a 20 mm parallel geometry with a stage gap of 400 μm; a solvent trap was placed over the apparatus to prevent evaporation from the gel. Frequency sweep experiments were performed at 25°C with 0.1% strain (within the linear viscoelastic region) over a frequency range of 0.1–100 rad s⁻¹.

*Table 1. Rheological storage modulus (*G'*) and loss modulus (*G''*) values for peptide hydrogels.*

<i>Peptide</i>	<i>G'</i> (Pa)	<i>G''</i> (Pa)
Ac-(ChaKChaK) ₂ -NH ₂	1325	199
Ac-FK(ChaK) ₃ -NH ₂	911	126
Ac- ^{F5} FK(ChaK) ₃ -NH ₂	638	79
Ac-IK(ChaK) ₃ -NH ₂	458	81
Ac-FK(^{F5} FK) ₃ -NH ₂	2091	161
Ac-IK(^{F5} FK) ₃ -NH ₂	615	90

Fig. 2. Representative TEM images of fibrils formed at 8 mM peptide in 200 mM NaCl solution. A. Ac-FK(^{F5}FK)₃-NH₂ peptide fibrils and B. Ac-FK(ChaK)₃-NH₂ peptide fibrils.

Fig. 3. Rheological frequency sweep data for: A. Modified Ac-(ChaKChaK)₂-NH₂ peptides and B. Modified Ac-(^{F5}FK^{F5}FK)₂-NH₂ peptides.

The rheological characterization data (Figure 3 and Table 1) was inconsistent with our previously published data [8]. In our previously published work, the Ac-(ChaKChaK)₂-NH₂ hydrogels were reported to have a weak storage modulus (G') of ~ 75 Pa (8 mM peptide, 200 mM NaCl). In contrast, in this study we found the Ac-(ChaKChaK)₂-NH₂ hydrogels to have a significantly stronger storage modulus of 1325 Pa. We found some significant differences in our experimental protocols that account for these differences. First, in our previously published work [8], the hydrogels were assessed after only several hours of maturation; in this work, the gels were allowed to stand for 24 h prior to rheological assessment. Second, in the previously reported data, the gels were applied to the rheometer stage by extrusion from plastic tubes: the gels were formed in plastic conical tubes, the bottom of the conical tubes were then cut off using razor blades, and the gel was finally squeezed from the tube onto the rheometer stage. In this study, a specialized pipettor that was designed for the handling of viscous materials was used to transfer the gels from plastic tubes onto the rheometer stage. The latter technique was shown to be much less disruptive to the mechanical integrity of the hydrogels. Thus, our previously published data showing that Ac-(ChaKChaK)₂-NH₂ hydrogels are relatively weak most likely report on gels in which the network is incompletely formed. Given enough time for maturation, the Ac-(ChaKChaK)₂-NH₂ hydrogels become significantly more rigid.

Modification of the *N*-terminal amino acid of these hydrogels did affect hydrogel rigidity. However, these effects did not strongly correlate to the aromaticity of the *N*-terminal residue as we predicted based on our postulations. As indicated above, the Ac-(ChaKChaK)₂-NH₂ hydrogels had a G' value of 1325 Pa. Peptides in which the *N*-terminal Cha residue was replaced with either Phe or F₅-Phe (Ac-FK(ChaK)₃-NH₂ and Ac-F₅K(ChaK)₃-NH₂) also formed fairly rigid gels with average G' values of 911 Pa and 638 Pa respectively. We predicted that these gels should be more rigid than the parent Ac-(ChaKChaK)₂-NH₂ hydrogels, which obviously is not true. In comparison, aromatic Ac-FK(F₅K)₃-NH₂ peptides form rigid hydrogels with G' values of 2091 Pa. When the *N*-terminus of this peptide or the Ac-(ChaKChaK)₂-NH₂ peptide was replaced with a nonaromatic Ile residue, the rigidity of the resulting hydrogels dropped significantly relative to the parent hydrogels. The Ac-IK(ChaK)₃-NH₂ and Ac-IK(F₅K)₃-NH₂ hydrogels had G' values of 458 Pa and 615 Pa respectively. While modification of the *N*-terminus does influence emergent hydrogel viscoelasticity, the trends do not strongly correlate with the aromaticity of the *N*-terminal amino acid.

This data provides insight into the relationship between the aromatic/hydrophobic character of amphipathic peptides and the emergent hydrogel properties of the assembled fibril networks. We postulated that the aromatic character of the *N*-terminal nonpolar residue potentially participates in specific noncovalent cross-linking interactions in hydrogel formation [8]. This hypothesis was based on observations from our previous work that showed a dramatic difference in hydrogel rigidity between amphipathic peptides in which the nonpolar residues are aromatic or nonaromatic; peptides with aromatic peptides tended to be more rigid [8]. However, data reported herein indicate that our previously reported data is likely a function of kinetically slower network formation (not fibril formation) for the nonaromatic peptides and not an inherent difference in specific fibril-fibril cross-linking effects. Given enough time, even the nonaromatic peptides form rigid hydrogels. In addition,

substitution of only the *N*-terminal residue of amphipathic peptides did not result in hydrogels in which the elasticity correlated strongly with the aromaticity of the *N*-terminal residue. The possibility remains that the differences are merely kinetic. That is, the fibril network that constitutes the hydrogels forms more slowly in the case of some of these peptides relative to the others. Perhaps all hydrogels approach a common maximum rigidity related to total fibril content if given enough time to mature. This work is ongoing and additional insight will be reported in due course.

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