Semiflexible Chain Networks Formed via Self-Assembly of β-Hairpin Molecules

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We report experimental results from a *de novo* designed oligopeptide that *intermolecularly* self-assembles into rigid hydrogel networks after an *intramolecular* folding event. Microscopy and neutron scattering reveal a fibril local structure that is approximately 3 nm in diameter and over several hundred nanometers in length. Oscillatory rheology suggests that the peptidic network viscoelastic behavior follows that theoretically predicted for heavily cross-linked, semiflexible polymer networks.

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The dynamics of semiflexible chains and the rheological properties of their networks have been rigorously studied both theoretically and experimentally [1–9]. This significant interest is due to the fact that biomacromolecules, such as F-actin [10], DNA [11,12], and proteins assemblies, exhibit semiflexible behavior. Studying the dynamics of these systems can lead to a better understanding of structure-function relations and *in vivo* behavior of biological systems. Microrheological techniques, including particle tracking, particle trapping, and diffusion wave spectroscopy and mechanical rheological techniques, have been employed on biological systems such as polymerized F-actin [2,13–16] bacteriophage fd [17], keratin [18], and polymerized microtubulin [19]. In this Letter, we introduce the first synthetic peptide analogue of cytoskeletal protein networks in which self-assembly (i.e., polymerization) occurs only after desired *intramolecular* peptide folding. The folded, β-hairpin peptides assemble into fibrils with a completely defined cross section on the nanoscale, unlike the complex, hierarchiacal assembly that occurs in other β-sheet-rich, fibril forming peptides. The well-defined polymerized networks display rheological properties predicted for semiflexible biopolymers with physical, permanent (i.e., not simply entangled) cross-link points. Since the system is based on non-natural, synthetic peptides, the results are not encumbered by experimental complications encountered in biological analogues during protein extraction from mammalian sources and subsequent purification. Thus the system provides a clean, synthetic model system to probe the physics of biopolymer systems.

The synthetic peptides, comprised of only 20 amino acids, are designed to *intramolecularly* fold under desired aqueous conditions and consequently *intermolecularly* self-assemble into supramolecular networks [20,21]. By linking network self-assembly to a proteinlike folding event, one can design dilute peptide solutions to undergo immediate, infinite viscosity changes to a self-supporting, rigid network with an array of aqueous environmental cues. These cues include physiological solution conditions or salt concentration, which is used as the folding stimulus in the work described herein. One of the ultimate goals of this controlled network formation is specific control of network rigidity and biocompatibility for tissue engineering applications. The peptide underlying the folding and self-assembly process described herein consists of the following amino acid sequence, $VKVKVKVK^{D}PPTKVKVKVKV-NH_2$, where V is valine [with side chain $CH(CH_3)_2$], K is lysine [with side chain $(CH_2)_4NH_2$], and P is proline (see Fig. 1 for the side chains). The central $^{D}P^{L}P$ dipeptide, where D and L represent the right- and left-handed isomers of proline, respectively, defines a type II′ β turn that enforces hairpin structure under folding conditions. In a *p*H 7.4 aqueous solution of low ionic strength, the peptide remains unfolded in a random coil conformation and the solution exhibits the low viscosity of pure water. However, on introducing a salt, electrostatic repulsions between like-charged lysine residues are screened and the turn sequence forces the arms of the peptide into a β-hairpin arrangement in which the arms are stabilized by intrastrand hydrogen bonding (Fig. 1). Once folded, the molecule exhibits facial amphiphilicity since the hydrophobic valine and hydrophilic lysine side chains are displayed from opposite faces of the folded backbone. The valine-rich faces of neighboring folded peptides consequently collapse together in order to exclude water and subsequently undergo further assembly into network fibril scaffolding via additional hydrogen bonding (Fig. 2, inset). The peptide solution thus undergoes an infinite viscosity change to a viscoelastic network. The ultimate moduli of the resultant networks are dependent on the concentration of stimulus. (The exact mecha-

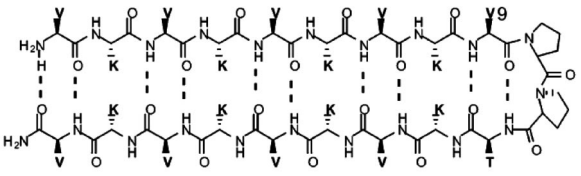

FIG. 1. 20 amino acid long sequence of the β-hairpin structure of the peptide.

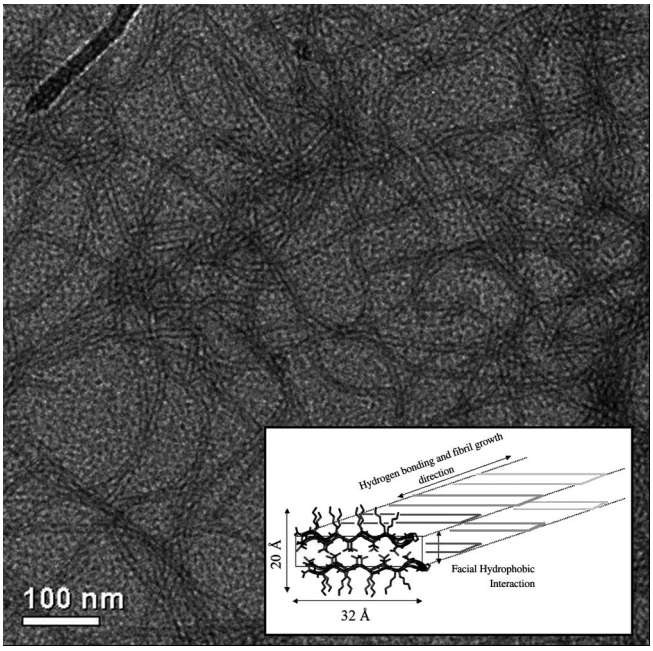

FIG. 2. TEM micrograph (negatively stained with uranyl acetate) of self-assembled fibrillar structures. Inset: The proposed self-assembled structure of the fibrils and the cross-section dimension.

nism of kinetic dependence of the folding and assembly process on folding stimulus concentration is currently under investigation.)

We have recently succeeded in the characterization of the nanoscale structure underlying the networks. The network scaffold is comprised of stiff, fibrillar structures with a completely defined, molecular cross section on the nanoscale. If one takes aliquots of gel and dilutes the network by a factor of ~20, the fibrils constituting the scaffold can be directly, individually observed. Figure 2 shows a transmission electron microscope (TEM) image of the negatively stained self-assembled structures. The fibrils are homogeneous in cross section, ~3 nm in diameter as directly measured from the TEM data, and can extend for over several hundred nanometers in length. The cartoon given in the inset of Fig. 2 shows the dimensions of the folded hairpin molecule and the proposed self-assembly structure of the fibrils. The hydrogen bonding direction corresponds to the long axis of the fibrils. The cross section of the fibrils consists of two β-hairpin molecules that are self-assembled via the hydrophobic interaction of the valine-rich faces. The width of the fibrils corresponds to the strand length of the folded peptide, which is approximately 3.2 nm. Importantly, the fibrils are not flexible but appear to be locally stiff. Since the sample was dried out prior to imaging, the absolute persistence length cannot be determined from the micrographs. The fibrils show no evidence of twisting or lateral packing that would produce polydispersity in fibril cross section. Both twisting and lateral packing have been observed for a number of β-amyloid and short-alternating sequenced peptides that, in general, irreversibly assemble into β-sheet fibrillar precipitates. However, due to the *intramolecular* folding mechanism, it was shown that the β-sheet formation of β-hairpin molecules studied here is reversible with both pH [20] and temperature [21].

Small-angle neutron scattering (SANS) was used to globally quantify the local structure of the underlying fibrils. Figure 3 shows the scattering curves and the cylinder form factor fits [22] for solutions with 0.6 and 6.1 mM peptide concentration. The good agreement between data and form factor fits indicates that the self-assembled fibrillar structures in Fig. 2 can be approximated by cylindrical geometries. The contour length of the fibrils is too large to be determined by SANS measurements. The fact that a -1 slope in the $\log(I)$ vs $\log(q)$ plot, where $q = (4\pi/\lambda) \times \sin(\theta/2)$ and θ is the scattering angle, is sustained at low q values reveals that these self-assembled chains behave as rigid rods even at low scattering vectors. Cylinder radii of 13.5 and 14.9 Å for the 6.1 and 0.6 mM peptide concentrations, respectively, as determined from the cylinder form factor fits agree very well with the fibril dimensions as imaged via TEM. The cross-sectional diameters of the fibrils can then also be determined from a modified Guinier analysis [23]. For 6.1 mM hydrogel the cross-sectional diameter was calculated as 30.9 ± 0.2 Å. This dimension is in excellent agreement with the width of the fibrils as seen in the TEM micrographs and is consistent with the cross-sectional diameter determined in similar fashion of hydrogels produced with 150 up to 600 mM of salt. The molar mass of the rod, m, is calculated as 479 Da/Å from the y intercept $I_c(q = 0)$ of Fig. 3 (inset) linear fit using $m = [I_c(q = 0)d^2N_A]/[\pi c(\rho_p - \rho_s)^2]$,

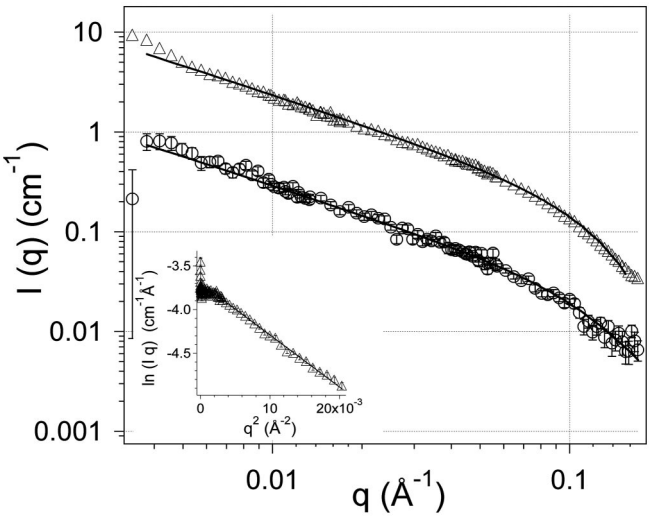

FIG. 3. SANS data for 6.1 mM (triangles) and 0.6 mM (circles) peptide solutions buffered at pH 7.4 with 50 mM bis-tris propane and 150 mM NaCl in D_2O. (The error bars for the 6.1 mM data are no larger than the symbols.) The solid lines are the cylinder form factor fits. Inset: Modified Guinier plot $\ln[I(q)q]$ vs q^2 for 6.1 mM peptide solution.

where d is the particle mass density, c is the concentration of the particles, and $(\rho_p - \rho_s)$ is the contrast between the scattering length densities of the particle and solvent. This is very close to the expected value (474 Da/Å) of the fibril cross section calculated using a model of folded β hairpins dimerized facially via hydrophobic assembly of the valine faces (Fig. 2, inset). The agreement of form factor fit and modified Guinier analysis, therefore, is indicative of a local facial dimmer character of the self-assembled structures and, in agreement with the TEM micrographs, strongly suggests that there is no interfibril lateral packing in the hydrogel networks. Similar analysis of the hydrogel samples with the same peptide but with different salt concentrations (400 and 600 mM NaCl) also indicates that this cross-sectional diameter is not affected by the ionic strength of the solution.

Hydrogels with different salt concentrations show significant differences in their rheological properties; an increase in ionic strength resulted in the formation of hydrogels with higher storage moduli. The consistency of the fibril nanoscale structure (all identical to Fig. 2) self-assembled at different salt concentrations suggests that the differences in elastic properties of the gels arise from the differences in network structure. Both TEM and SANS data suggest that the network is formed of semiflexible chains. Semiflexible network elasticity theories can be used to define the characteristic chain and network dimensions of the system. Figure 4 shows the scaling relationship between the peptide concentration and the plateau modulus of the hydrogels. The inset of Fig. 4 shows frequency sweep data for the 3.1 (~7 mg/ml), 4.6, 6.1, and 9.2 mM peptide concentrations. For all gels the G' (storage modulus) values are an order of magnitude greater than their corresponding G'' (loss modulus) values, exhibiting no crossover within the 0.1–10 rad/s frequency range. In addition, G' values are insensitive to frequency. This kind of rheological behavior suggests that the cross-link points of the fibrils are permanent junction points and not simply entanglements. In addition, we have found that the maximum strain that the material can withstand increases with decreasing peptide concentration (data not shown). This behavior is also predicted by Mackintosh *et al.* [1] and attributed to permanent junction points. It is important to note here that the β-hairpin hydrogels are purely physical networks and no chemical cross-linking strategy is involved, in either the formation of supramolecular fibril structure or the fibril cross-link points. Because of the lack of direct observation, the exact self-assembled nanostructure of these cross-links is still unknown. However, evidence to date suggests that they are formed by ''defects'' in the hydrophobic collapse of valine faces between different peptides of a growing fibril; i.e., the hydrophobic collapse does not form a perfect dimmer between peptides and provides for a new fibril axis away from the parent fibril. The plateau moduli of the hydrogels scale with the peptide concentration with an exponent of 2.5($G' \propto c^{2.5}$).

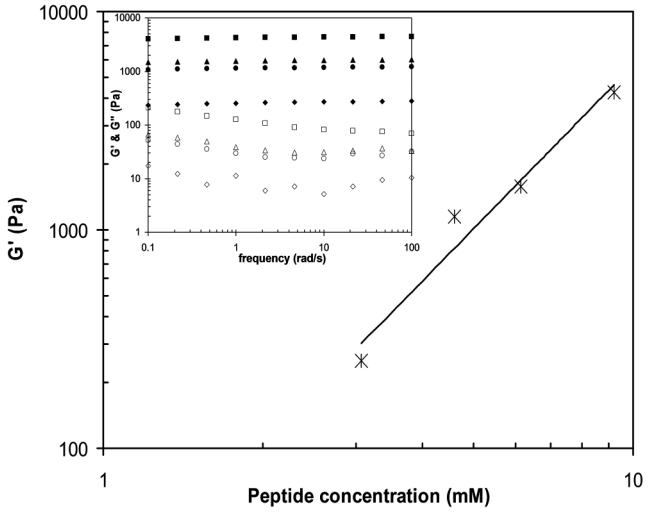

FIG. 4. Storage modulus (G') vs peptide concentration. The solid line represents the fit to data points (equation of the line $G' = c^{2.48}$, $R^2 = 0.96$). Inset: Frequency sweep data for 3.1 (◆), 4.6 (●), 6.1 (▲), and 9.2 (■) mM peptide solutions (pH 7.4, 50 mM bis-tris propane, 150 mM NaCl, 25 °C). Solid and open symbols represent the storage (G') and the loss (G'') modulus, respectively.

This scaling is in agreement with the Mackintosh theory [1], which was proposed for densely cross-linked gels. This theory predicts the plateau modulus as

$$G' \sim \frac{\kappa^2}{kT}(ac)^{5/2}, \tag{1}$$

where κ is the semiflexible chain bending modulus, a is the monomer size, and c is the concentration. The bending modulus is related to the persistence length, l_p, with $\kappa \sim l_p kT$. The persistence length of the self-assembled fibrils can be estimated using the scaling relationship given in expression (1). For 6.1 mM peptide concentration, the measured G' value is approximately 1600 Pa. This leads to an l_p value of approximately 55 nm. This is in qualitative agreement with the TEM micrographs. Also, Aggeli *et al.* estimated similar l_p values for self-assembled β-amyloidlike structures formed from short peptide sequences [24]. The characteristic mesh size, ξ, of the network can be approximated as 34 and 20 nm for 3.1 and 9.2 mM peptide concentrations, respectively, using the $\xi \sim 1/\sqrt{ac}$ expression for stiff chains. As expected, the ξ of the network is small compared to the l_p of the chains.

Microrheological studies performed on F-actin [13] at relatively dilute concentrations and bacteriophage fd [17], which both form an entangled chain network structure, reveal plateau moduli with much weaker dependencies on peptide concentration. The rheological properties of these systems are in agreement with the predictions of Isambert and Maggs [2] and Morse [7]. In their works, $G' \sim c^{1.4}$ and $G' \sim kT/l_e\xi^2$, where entanglement length $l_e \sim \xi^{4/5} l_p^{1/5}$. When these relations are used for estimating the l_p for the β-hairpin fibrils in a 6.1 mM peptide hydrogel

network, it is calculated to be less than 1 Å. This is not a reasonable value for the observed self-assembled structure. The peptide hydrogels exhibit rheological behavior very similar to that predicted for cross-linked networks. The fibrils do not freely undergo tangential motion as in F-actin networks. Thus, the Mackintosh theory gives a better description of the system at the studied low frequencies.

We have introduced the first synthetic peptide system that displays the rheological scaling behavior predicted for natural, semiflexible biopolymer networks. The exact nanostructure of the self-assembled peptide fibrils, combined with the purity of a synthetic chemical system, provides a model semiflexible biopolymer system with which to explore fundamental predictions of theory through appropriate synthetic peptide design and assembly. For example, the arms of the β hairpins can be made shorter or longer in order to decrease or increase, respectively, the cross section of the resulting fibrils, which, in turn, might change the bending modulus and persistence length of the self-assembled chains. The homogeneity of the self-assembled fibril nanostructure is crucial for comparison to natural protein systems since chain cross sections are completely defined in natural, semiflexible cytoskeletal proteins such as F-actin and microtubulin or natural viral assemblies such as bacteriophage fd. In addition, the fibril persistence length and, thus, bending modulus may be controllably increased by the introduction of covalent bonding between hairpins along the fibril axis. This can be accomplished by the simple incorporation of non-natural amino acids with cross-linkable functional groups. Therefore, synthetic peptides designed to intramolecularly fold and consequently self-assemble provide a new, tailorable equivalent to the absolute defined nanostructure of natural self-assembling protein systems.

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[22] For the cylinder form factor fits, the following function was used:

$$I(q) = \frac{\phi}{V_{\mathrm{cyl}}} \int_0^{\pi/2} f^2(q,\alpha) \sin\alpha d\alpha, \; f(q,\alpha) = 2(\rho_{\mathrm{cyl}} - \rho_{\mathrm{solv}}) V_{\mathrm{cyl}} \frac{\sin(qH\cos\alpha)}{qH\cos\alpha} \frac{J_1(qr\sin\alpha)}{(qr\sin g\alpha)}.$$

The volume fraction (ϕ) of the peptide and the scattering length contrast $(\rho_{\mathrm{cyl}} - \rho_{\mathrm{solv}})^2$ were fixed. The outputs of the fit are cross-sectional diameter and the length of the cylinders. The scattering length density of the peptide is calculated as 2.66×10^{-6} Å^{-2} from the chemical structure of the peptide, $C_{107}N_{29}O_{21}H_{163}D_{37}$, assuming that all exchangeable protons are replaced with deuterium and the density of the peptide is 1.35 g/ml.
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